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# The Journal of Laboratory and Clinical Medicine

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# PROTEIN REQUIREMENTS OF ADULTS

D. M. Hegsted, Ph.D., A. G. Tsongas, M.P.H., D. B. Abbott, M.P.H., and F. J. Stare, Ph.D., M.D.
Boston, Mass.

## INTRODUCTION

RECENT surveys on the nutritive state of population groups have clearly demonstrated that the intake of many nutrients at levels considerably below National Research Council recommendations are compatible with apparent good health.[1-4] Estimates of absolute requirements such as have been arrived at for thiamine[5] are needed if correlation with clinical findings and nutrient intake is to be found, or if dietary intakes are to be more helpful in assessing nutritive state. Also, levels approaching requirement should form the first goal to be sought in rehabilitation work, when food supplies are limited, rather than optimum levels which, under such conditions, may be wasteful.

The recommended allowance of the National Research Council for protein, 1 Gm. per kilogram of body weight per day, is easily obtainable on diets containing foods of animal origin. It is generally conceded that there is a safety factor of about 50 per cent in this allowance, presumably to allow for differences in nutritional value of various proteins. However, a considerable portion of the population (see preceding reviews) consumes less protein than the allowance mentioned, usually of the so-called poor quality proteins, but without ill effects as revealed by clinical examination. Minimum levels of protein, related to the nutritional value of the diet, are needed if dietary surveys are to be used in assessing the relative adequacy of the proteins in ordinary diets.

In view of the fact that there is a paucity of data upon the protein requirements of adults on mixed diets, we have studied nitrogen balance on twenty-six adults who were fed diets low in protein and devoid of animal protein and have investigated the effect of replacing part of the protein in the diet by meat, soy flour, wheat germ, and white bread.

## REVIEW OF LITERATURE

Several reviews are available covering protein requirements for adults and the individual papers need not be reviewed. It should be mentioned, however, that in evaluating the nutritional value of the proteins in a diet by nitrogen balance studies, the level of nitrogen fed should be sufficiently low to produce negative balance and should be fed sufficiently long to assure a reasonably constant nitrogen output. Since there is a lag in the change of nitrogen excretion with decreases in dietary nitrogen, short periods will show negative

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balances which are too large. Thus, if either of these conditions is not met, the determined requirement for nitrogen will be too high.

Leitch and Duckworth[6] estimated the average human requirement to be approximately 50 Gm. of protein per day. This estimate was reached by plotting selected nitrogen balance figures from the literature against protein intake. A regression line was drawn through the positive balances and another through the negative balance figures. The point at which these lines were equidistant from the line of balance was taken as the estimated requirement. The principal criterion of suitability of the data used was that the subjects received the same diet for at least three days preceding the collection of urine and stools. The data were from many sources and no consideration was made of the size of the subjects or the quality of the protein fed. Many of the data show negative balances at such high levels of nitrogen intake that they must be discarded on a logical basis as either inaccurate or unrepresentative of normal adults or usual diets. Further, three days is often inadequate for obtaining a stabilized nitrogen output (see Selection of data). Finally, relatively few data are available at critical levels of intake and practically none at really low levels. Thus the spread of the data is so limited at the levels of real interest as to limit seriously the use of the method employed. The estimate of 50 Gm. of protein per day is undoubtedly high.

The data of Sherman and associates[7] and of Hindhede[8] are extensive and approach the criteria of an adequate study, although many of the data were collected on the same subjects and the level of nitrogen intake was usually so adjusted as just to produce negative balance. Thus the spread of the data is limited. By rigorous selection of data, which appears justified, Sherman estimated the protein requirement to be about 40 Gm. per day for a man weighing 70 kilograms. Many of the data, however, were collected on diets consisting almost wholly of a single food—potatoes or wheat or corn. Thus this estimate may be high due to the supplementary action of the proteins of mixed diets.

With regard to supplementary action, Sherman's data clearly show improvement in the nutritive value of cereal diets by the addition of small amounts of milk. However, the differences were apparently not sufficiently large to allow separate estimates of the requirement on the two regimens (cereal alone and cereal plus milk), and data on both types of diets were used in arriving at the estimated requirement of 40 Gm. Considering the levels of nitrogen fed, little justification for recommending high quality proteins can be found in these papers.

Estimates of protein requirements for adults have been arrived at from consideration of the endogenous nitrogen in the urine on protein-free diets. Brody[9] has made an extensive compilation of the data available and concludes, in agreement with Terroine and Sorg-Matter[10, 11] and Smuts,[12] that there is a parallelism between basal energy metabolism and endogenous nitrogen excretion. The minimum urinary nitrogen excretion for species ranging from mice to dairy cows appears to be approximately 2 mg. nitrogen (12.5 mg. conventional protein) per calorie of basal heat expended. As pointed out by

Smuts[12] and Terroine[13] this minimum protein requirement should be replaceable, gram for gram, by a protein with a biologic value of 100 which is completely digestible. On this assumption, the average adult requirement for this high quality protein would be approximately 19 Gm. If one assumes a biologic value as low as 50, the requirement would be satisfied by approximately 38 Gm. Incomplete digestion would similarly raise the requirement. However, few proteins which have been studied have either biologic values or digestibilities as low as 50.[14] On a theoretical basis, then, the requirement is probably between 20 and 40 Gm. In fact, Terroine[13] concludes that the consumption of a diet of natural foods, rice alone, for example, will adequately cover the protein needs when the caloric needs are satisfied.

The endogenous nitrogen excretion of rats was also found proportional to basal caloric expenditure by Ashworth and Brody.[15] The average figure given by these authors is 1 mg. of nitrogen per 0.71 calories or approximately 1.4 mg. of nitrogen per basal calorie. This figure may be more nearly correct than that of Smuts since it was arrived at using long depletion periods. Protein requirements based on this estimate would thus be approximately 30 per cent less than those given. In this paper, however, we have used Smuts's estimate principally for conservative reasons and because it is based on several species.

## INVESTIGATIONS

*Subjects.*—The subjects used in these studies were twenty-six apparently healthy adults ranging in age from 19 to 50 years. They included five medical students, five home economic students, three graduate nurses, nine students from the Harvard School of Public Health, and four research assistants. Three participated in two different studies. They were all volunteers but were paid while on the study. All of the subjects were selected by personal interview with special consideration for trustworthiness since they continued their regular activities and could not be supervised other than at mealtimes. Each subject collected all urine and stools during the investigation and these were delivered at mealtimes to the investigators in charge. Aliquots of the urine were taken each day and combined. Kjeldahl analyses were made every two, three, or four days, depending upon the investigation. All stools were homogenized with water in a Waring Blender and aliquots handled in the same manner as the urine.

*Diets.*—All of the diets used in these studies, with the exception of Diet H, were based upon the basic diet, Diet IB, shown in Table I. This diet is devoid of animal protein and is referred to as the all-vegetable diet. The constituents of this diet were selected to approximate in some respects certain low-cost diets. Thus 50 per cent of the nitrogen was supplied by white bread (containing no skim milk solids) and a total of 62 per cent was supplied by cereal products. Potatoes supplied about 13 per cent of the nitrogen, and the total from vegetable products was approximately 30 per cent. The remaining 8 per cent came from fruits. Individual servings of each food were weighed daily and remained constant on each diet. However, considerable ingenuity

TABLE I. PROTEIN CONTENT OF DIETS

| FOOD | BASIC ALL-VEGETABLE DIET IB | | | | DIET IIB | | | | DIET H | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | AMOUNT | PROTEIN | | | AMOUNT | PROTEIN | | | AMOUNT | PROTEIN | | |
| | (GM.) | (GM.) | PER CENT | | (GM.) | GM. | PER CENT | | (GM.) | GM. | PER CENT | |
| Bread (no milk solids) | 144 | 12.000 | 50.0 | 50.0 | 96 | 8.000 | 33.3 | 33.3 | – | – | – | – |
| Rice | 24 | 1.824 | 7.6 | 12.0 | 16 | 1.216 | 5.1 | 8.0 | – | – | – | – |
| Yellow corn meal | 12 | 1.056 | 4.4 | | 8 | 0.704 | 2.9 | | 15 | 1.320 | 17.1 | 17.1 |
| Potatoes | 160 | 3.200 | 13.3 | 13.3 | 107 | 2.140 | 8.9 | 8.9 | – | | | |
| Onions | 40 | 0.560 | 2.3 | 16.3 | 27 | 0.378 | 1.6 | 11.0 | 50 | 0.700 | 9.1 | 60.8 |
| Carrots (canned) | 160 | 1.600 | 6.7 | | 107 | 1.070 | 4.5 | | 200 | 2.000 | 25.9 | |
| Tomatoes (canned) | 80 | 0.800 | 3.3 | | 53 | 0.530 | 2.2 | | 80 | 0.800 | 10.3 | |
| Lettuce | 80 | 0.960 | 4.0 | | 53 | 0.636 | 2.7 | | 100 | 1.200 | 15.5 | |
| Applesauce (canned) | 80 | 0.160 | 0.7 | 8.4 | 53 | 0.106 | 0.4 | 5.5 | 300 | 0.600 | 7.8 | 22.1 |
| Orange juice | 160 | 1.440 | 6.0 | | 107 | 0.963 | 4.0 | | – | | | |
| Peaches (canned) | 80 | 0.400 | 1.7 | | 53 | 0.265 | 1.1 | | 100 | 0.500 | 6.5 | |
| Pears (canned) | – | | | | – | | | | 200 | 0.400 | 5.2 | |
| Olives | – | | | | – | | | | 20 | 0.200 | 2.6 | |
| Meat | – | | | | 34 | 7.990 | 33.3 | 33.3 | | | | |
| Total (Calculated) | | 24.000 | 100.0 | 100.0 | | 23.998 | 100.0 | 100.0 | | 7.720 | 100.0 | 100.0 |

was used in preparing these same foods into dishes which were tasty, interesting, and varied. For example, the corn meal was sometimes served as cereal, sometimes as topping for a vegetable casserole, and sometimes as baked mush with tomato sauce. Individual casseroles were used throughout in preparing dishes which could not be accurately measured after cooking.

The total caloric content of Diet IB was calculated to be 950 calories per day. Additional calories to maintain weight were supplied in the form of rendered butter, cooking oils, cornstarch biscuits, Coca-Cola, ginger ale, jelly, Karo syrup, sugar, and prepared candies. The subjects were allowed these foods freely, but everything eaten was recorded daily. This permitted estimates of the total caloric intakes. The figures for nitrogen intake were also adjusted to include the small amount of nitrogen in these caloric supplements as determined by analysis.

Each day two extra meals were prepared. These meals were placed in waxed cardboard containers in the refrigerator and were homogenized with water the next day in the Waring Blender. Aliquots were saved for Kjeldahl analysis. Figures on nitrogen intake were arrived at from the nitrogen content of the meals to which was added the nitrogen in the caloric supplements. The amount of nitrogen in these supplements was small and therefore each subject, regardless of weight, had essentially the same nitrogen intake. Some coffee, prepared from concentrates to avoid daily analyses, was allowed. This nitrogen was considered to have no nutritive value. It was not included in the calculations of dietary nitrogen and an equivalent amount was subtracted from the urinary nitrogen figures.

Diet IIB (Table I) represents Diet IB supplemented with meat. The amount of *each* food in Diet IB was decreased by one-third and sufficient meat was added to supply approximately the nitrogen thus removed. Thus, the nitrogen

intake was approximately the same on Diets IIB and IB but about one-third was supplied by meat in Diet IIB. Daily dietary analyses were made as before. The substitution of meat for one-third of the basic diet resulted in a considerable decrease in the caloric content of the diet. This loss was covered by additional amounts of the caloric supplements.

TABLE II. SOURCE AND AMOUNT OF PROTEIN SUPPLIED IN VARIOUS DIETS COMPARED WITH BASIC ALL-VEGETABLE DIET IB

| DIET | IB (PER CENT) | MEAT* (PER CENT) | SOY FLOUR† (PER CENT) | WHEAT GERM‡ (PER CENT) | BREAD (PER CENT) | TOTAL AMOUNT AS PER CENT OF IB |
|---|---|---|---|---|---|---|
| IB | 100.0 | | | | | 100 |
| IA | 125.0 | | | | | 125 |
| IC | 150.0 | | | | | 150 |
| IIB | 66.6 | 33.3 | | | | 100 |
| IID | 66.6 | | 33.3 | | | 100 |
| IIE | 66.6 | | | 33.3 | | 100 |
| IIC | 66.6 | | | | 33.3 | 100 |
| G | 56.0 | 28.3 | | | | 85 |
| F | 100.0 | 220.0 | | | | 320 |

*Ground top of the round prepared in various ways. Thanks are due to Mr. J. H. McManus, Regional Chairman of the American Meat Institute for making arrangements for the purchase of meat.

†Stoy, used in biscuits, provided by the A. E. Staley Manufacturing Company, Decatur Ill.

‡Used in biscuits, provided by the Battle Creek Foods, Battle Creek, Mich.

Other diets used are shown in Table II. As can be seen, Diets IA and IC were of the same percentage composition as Diet IB, but each constituent (Table I) was increased 25 per cent and 50 per cent, respectively. Diets IIC, IID, and IIE were the same as Diet IIB (Table I) with bread, soy flour, and wheat germ, respectively, replacing the meat as a source of supplementary protein. Diet G was similar to Diet IIB containing meat, but each constituent including the meat was decreased approximately 15 per cent. Diet F was a high protein diet, IB plus 300 Gm. of meat. Diet H, shown in Table I, was fed at the beginning of Experiment 5 as a preliminary depletion diet. It supplies approximately 8 Gm. of protein per day. Diets J and K consisted of Diet IB plus varying amounts of glycine and cystine. These diets were used in part of Experiment 5 but will not be discussed here as they were concerned with another aspect of this study which will be reported later. Care was taken in weighing the foods, and the nitrogen content seldom varied as much as 0.2 Gm. from the mean value for the experiment.

The vitamin and mineral content of the diets was maintained by supplements* and, with the exception of riboflavin and calcium, was above the National Research Council's recommended daily allowances. Studies on riboflavin and calcium metabolism were made on these diets and will be reported later. The calcium intake varied from 400 to 1,000 mg. per day; the riboflavin intake, from 1.5 to 3.3 mg. per day.

*The supplements included thiamine, riboflavin, nicotinic acid, calcium, phosphorus, iron, and copper. Thanks are due to Abbott Laboratories, North Chicago, Ill., to Merck & Company, Inc., Rahway, N. J., and to United Chemical and Organic Products, Chicago, Ill., for these materials.

*Plan of Investigations.*—The general plan of the investigations is shown in diagrammatic form in Fig. 1. In most of the studies, the collection of stools and urine was preceded by a three-day adjustment period on the diet and a one-day period between the different diets. In all studies except Experiment 4, three collections of two days or more were obtained on each diet.

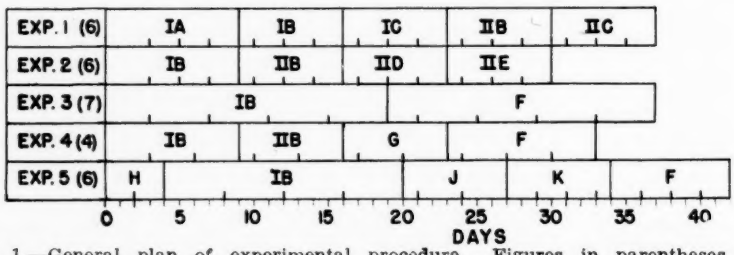


Fig. 1.—General plan of experimental procedure. Figures in parentheses represent number of subjects on each experiment. Small bars show the days included in each analysis of urine and stools.

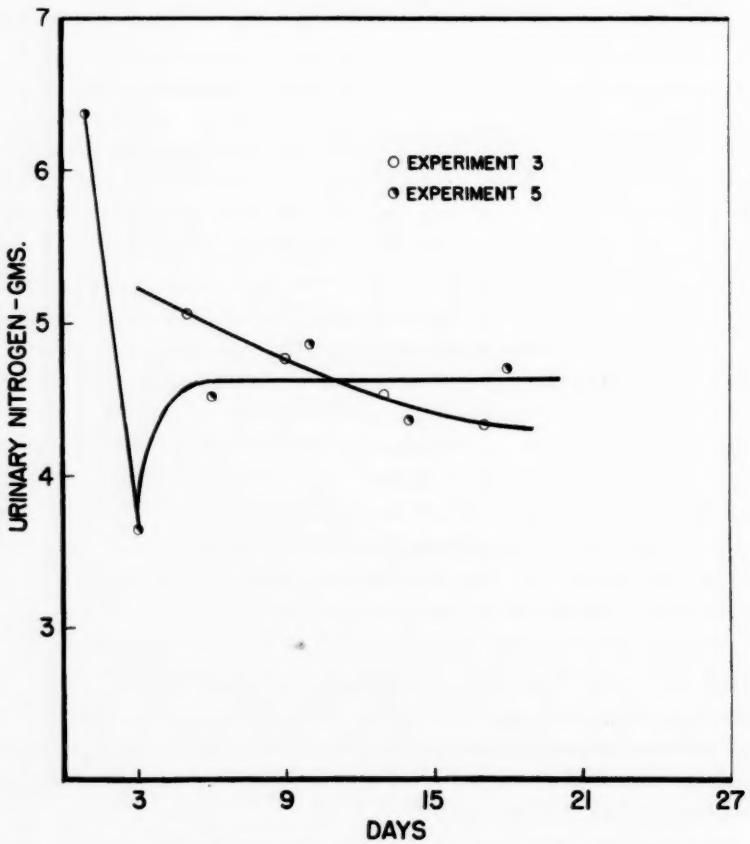


Fig. 2.—Average urinary nitrogen excretion in Experiments 3 and 5.

*Selection of Data.*—It was apparent from the data, especially in Experiment 3, that there was a great difference in subjects in the time required for stabilization on the diet. Of the seven subjects in Experiment 3, four showed a more or less regular fall in urinary nitrogen per day in the four four-day collection periods amounting to a difference of more than 1 Gm. per day at the start and at the end. The output of one subject was essentially constant and differed only 0.2 Gm. per day at the start and the end. The remaining two fell slightly, showing less than 0.5 Gm. per day difference in the first four days compared with the last four. The average urinary nitrogen excretion of the seven subjects is shown in Fig. 2. Also shown in this figure are the results of urinary excretion in Experiment 5 in which the subjects were fed the low nitrogen diet, Diet H, for four days before receiving the basic diet, Diet IB. Although the results are less consistent than in Experiment 3, it is apparent that there is no tendency for the urinary nitrogen to fall after this depletion period.

In the light of these results, it is certain that three days is far from sufficient for many subjects to reach a stabilized nitrogen output unless a preliminary very low nitrogen period is used. Thus it is recognized that much of the data in the literature probably show urinary nitrogen figures too high for the nitrogen fed and, similarly, that some of our subjects were undoubtedly not stabilized sufficiently. We have attempted to correct this somewhat by using the urinary nitrogen figures during the last four days of those subjects on Diet IB, if these periods were considerably lower than the preceding ones. However, the average fecal nitrogen of the total collection period was always used. Since the supplemented diets always followed the basal diet, we have not selected the data in these periods. It is recognized, therefore, that our estimate of protein requirement may be somewhat *high* and that the order of the experiments places the basic all-vegetable diet, Diet IB, in a less favorable position compared with the supplemented diets.

## RESULTS

The results of all of the nitrogen balance studies on the all-vegetable Diet IB are plotted against body weight, surface area, nitrogen intake per kilogram of body weight, and nitrogen intake per square meter of body surface* in Figs. 3 to 6. Both regression lines, one for the best estimate of X (values on the abscissa) and the other for the best estimate of Y (values on the ordinates), are shown. Both regression lines in Fig. 6 are used later in the paper, although in most instances Equation 1 (Fig. 6) is used since we are interested in estimating the value on the abscissa (body size or nitrogen intake) at zero balance.†

It will be recalled that the all-vegetable diet supplied approximately 4.5 Gm. of nitrogen per day (28 Gm. of protein) regardless of body size. This

*Taken from the DuBois body surface chart for height and weight as modified by Boothby and Sandiford (Duncan, G. G.: Diseases of Metabolism, Philadelphia, 1942, W. B. Saunders Co.).

†The standard error of the estimate of x ($\sigma$ s x) is given in Figs. 3 to 9. The two small bars on the line of zero balance about regression line 1 show the relative magnitude.

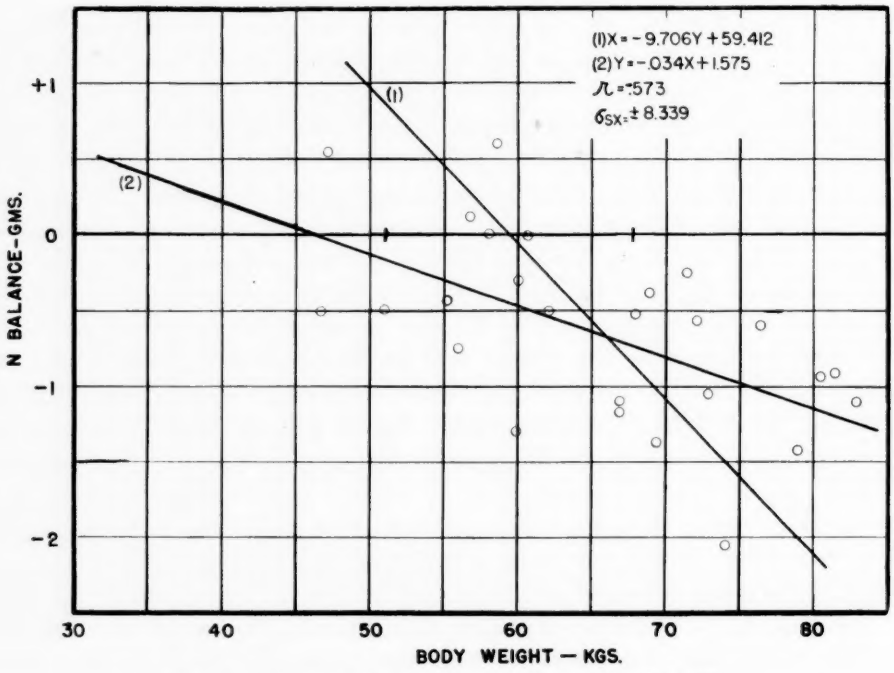


Fig. 3.—Nitrogen balance versus body weight—Diet IB.

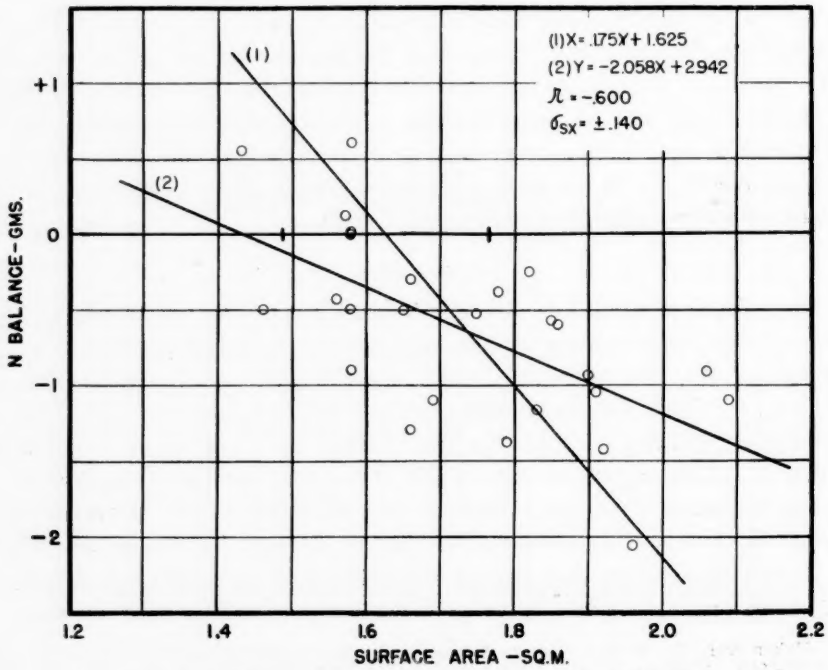


Fig. 4.—Nitrogen balance versus surface area—Diet IB.

value varied slightly with individuals and in different experiments. Thus, one expects a better correlation if intake is calculated per unit of body size. Actually, the correlation is not much improved if calculated per unit of body weight but is considerably better if intake per unit of body surface is used.

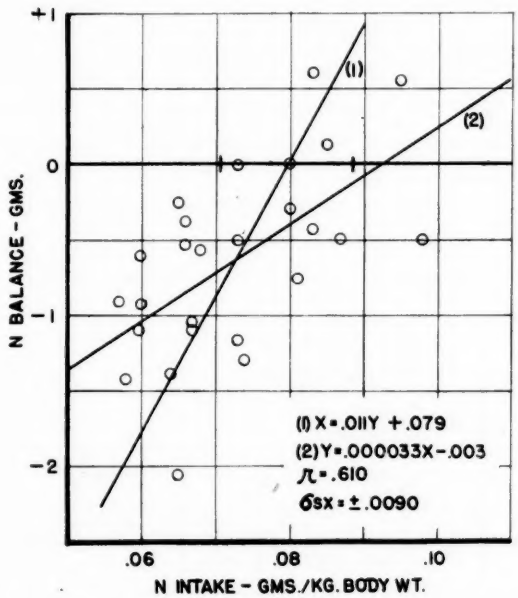


Fig. 5.—Nitrogen balance versus nitrogen intake per kilogram of body weight—Diet IB.

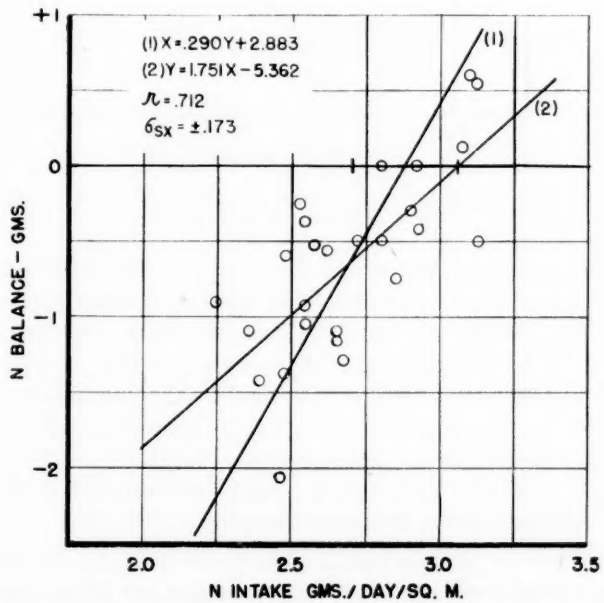


Fig. 6.—Nitrogen balance versus nitrogen intake per square meter of body surface—Diet IB.

The correlation coefficient in the latter instance (Fig. 6) is 0.71 compared with 0.61 on a body weight basis (Fig. 5). Intake per square meter of body surface is thus considered the most appropriate unit in considering protein requirements and is used throughout this paper. This is in agreement with the previous findings of Terroine and Sorg-Matter,[10] Ashworth and Brody,[15] and Smuts.[12] It is realized that the curves cannot be extended unduly. Increases in positive balance will not, for example, continue indefinitely.

According to Equation 1 in Fig. 6, the estimated intake at zero balance is 2.88 ± 0.17 Gm. of nitrogen per square meter of body surface. A man whose weight is 70 kilograms and height, 160 centimeters (surface area 1.73 square meters) would thus require between 4.68 and 5.27 Gm. of nitrogen per day from the all-vegetable diet, Diet IB, which corresponds to from 29.3 to 32.9 Gm. of conventional protein per day. A man of similar weight but 183 centimeters

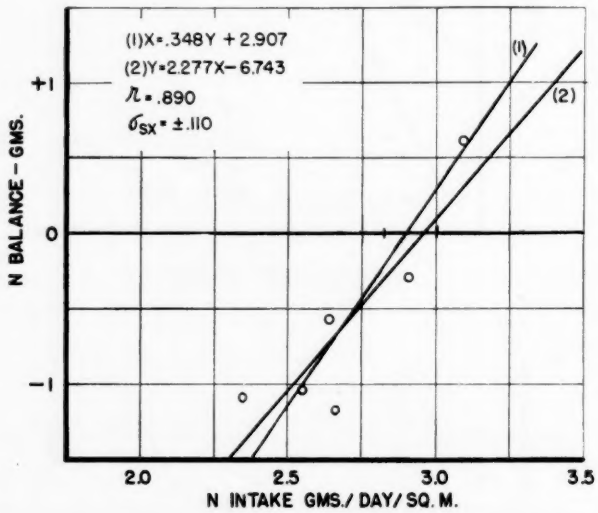


Fig. 7.—Nitrogen balance versus nitrogen intake per square meter of body surface of subjects in Experiment 5—Diet IB.

in height (1.91 square meters) would require from 34.5 to 38.6 Gm. of protein per day. It is of interest to compare the figures obtained on all the subjects with those in Experiment 5 where the subjects were apparently depleted by the period on Diet H. These results are plotted in Fig. 7. The correlation is good although the number of subjects is small. The requirement here is found to be 2.91 ± 0.11 Gm. per square meter of body surface per day which is essentially the same figure found. It appears, therefore, that the requirement arrived at in the previous data is not greatly overestimated, although ideally the studies should have been of longer duration.

The data obtained in Diet IIB, in which one-third of the protein was supplied by meat, are plotted against nitrogen intake per square meter of surface area in Fig. 8. This line crosses the line of balance at 2.73 Gm. nitrogen per square meter, which would indicate a protein requirement of 29.5 Gm. for a man

with a body surface of 1.73 square meters. However, the correlation is poor and many of the subjects were in positive balance, some without appreciable depletion during the preceding period on Diet IB. We may thus expect this estimate to be high and better results would have been obtained at a lower nitrogen intake. This was done in Experiment 4 using Diet G. The data on these subjects, who had been on the experimental diet for sixteen days previously, are plotted in Fig. 9. Only one regression line is shown since the two

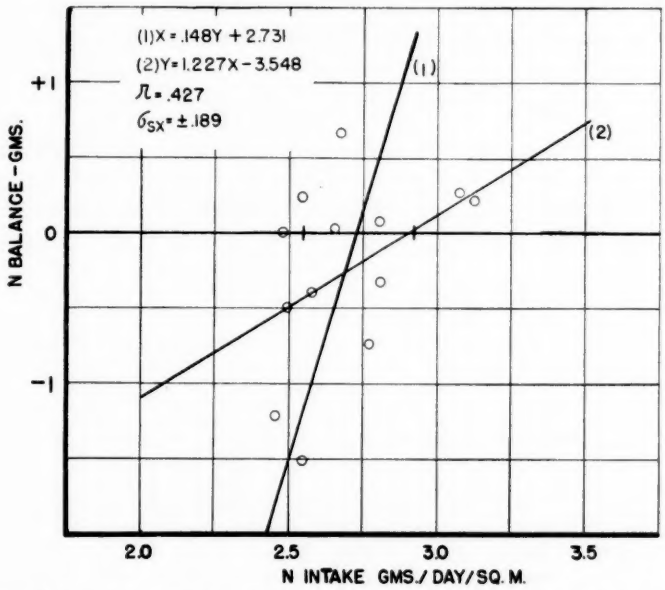


Fig. 8.—Nitrogen balance versus nitrogen intake per square meter of body surface—Diet IIB.

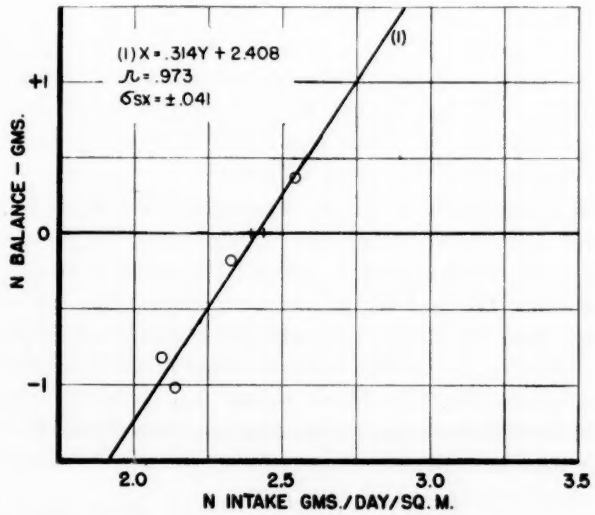


Fig. 9.—Nitrogen balance versus nitrogen intake per square meter—Diet G.

are practically identical. The estimated requirement is 2.41 ± 0.04 Gm. nitrogen per square meter. Although based on only four subjects, this estimate would give a protein requirement of from 25.7 to 26.5 Gm. per day for a man with a body surface of 1.73 square meters. We believe this estimate to be more nearly correct for the diet containing meat.

Insufficient data are available to calculate the comparable requirements on Diets IIC, IID, and IIE. We show rather a summary of the data in Table III where the balances obtained are compared with the balance which would result from the same intakes on Diet IB as calculated by equation 2 derived from the data in Fig. 6:

$$\text{Balance} = 1.751 \text{ Intake per square meter} - 5.362$$

The variations in balance found in the different diets and those obtained on Diet IB were compared by Fisher's "t" test. It is seen that the improvement on Diet IIB is highly significant. There can thus be no doubt that the diet with meat is an improvement over the all-vegetable diet. It appears likely

TABLE III. NITROGEN BALANCE ON SUPPLEMENTED DIETS COMPARED WITH BASIC ALL-VEGETABLE DIET

| DIET | SUPPLEMENT | WEIGHT (KG.) | AREA (SQUARE METER) | URINARY NITROGEN (GM.) | FECAL NITROGEN (GM.) | AVERAGE NITROGEN INTAKE | | N BALANCE (EXPERIMENTALLY DETERMINED) (GM.) | CALCULATED N BALANCE ON DIET IB AT SAME INTAKE† (GM.) | DIFFERENCE (GM.) | LEVEL OF SIGNIFICANCE* ("P") |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | GM. | GM. PER SQUARE METER | | | | |
| IB | None | 65.5 | 1.73 | 4.29 | 1.04 | 4.70 | 2.72 | −.63 | −.60 | −.03 | − |
| IIB | Meat | 63.5 | 1.69 | 3.87 | 0.90 | 4.53 | 2.68 | −.24 | −.67 | +.43 | <.001 |
| IIC | Bread | 60.2 | 1.64 | 4.33 | 0.83 | 4.65 | 2.83 | −.51 | −.41 | −.10 | >.9 |
| IID | Soy flour | 60.7 | 1.67 | 4.07 | 1.09 | 4.27 | 2.56 | −.89 | −.88 | −.01 | >.9 |
| IIE | Wheat germ | 60.7 | 1.67 | 3.43 | 1.02 | 4.34 | 2.60 | −.11 | −.81 | +.70 | .1 |

*Probability of difference from Diet IB being due to chance.
†From Equation 2 in Fig. 6.

that Diet IIE (wheat germ) would also show significant improvement if more data were available, although in the data at hand the differences are on the borderline of significance. Soy flour and bread apparently do not improve nor depress nitrogen balance in these experiments.

*Digestibility.*—As shown in Fig. 1, three diets, IA, IB, and IC, which were of identical percentage composition but which varied in amount, were fed to six of the subjects in Experiment 1. Diet IA contained 25 per cent more of each constituent than IB, and IC, 50 per cent more. The differences in fecal nitrogen on these three diets allow an estimate of the digestibility of the nitrogen in the diet. In Fig. 10 is shown the average fecal nitrogen on these three diets. It is fortuitous that the three points fall exactly on a straight line. An extension of the line to zero nitrogen intake may be taken as an estimate of the metabolic nitrogen (fecal nitrogen on a nitrogen-free diet) of these subjects whose average weight was 61 kilograms.

If 0.395 Gm. be taken as the metabolic nitrogen, then the excess nitrogen excreted was 0.950 − 0.395 = 0.555 Gm. on Diet IB, 0.710 on Diet IA, and

TABLE IV. DIGESTIBILITY OF PROTEIN SUPPLEMENTS AND OF SUPPLEMENTED DIETS

| | DIET | | | | |
|---|---|---|---|---|---|
| | IIB | IIC | IID | IIE | F |
| Supplement | Meat | Bread | Soy flour | Wheat germ | Meat |
| Total N intake (Gm.) | 4.53 | 4.65 | 4.27 | 4.34 | 14.7 |
| Estimated N from Diet IB | 3.00 | 3.00 | 3.00 | 3.00 | 3.00 |
| Estimated N from supplement | 1.53 | 1.65 | 1.27 | 1.34 | 11.7 |
| Average fecal N (Gm.) | 0.90 | 0.83 | 1.09 | 1.02 | 1.55 |
| Fecal N from Diet IB (Fig. 10) | 0.77 | 0.77 | 0.77 | 0.77 | 0.77 |
| Fecal N from supplement | 0.13 | 0.06 | 0.32 | 0.25 | 0.78 |
| Digestibility of supplement (per cent supplement absorbed) | 91.5 | 96.4 | 74.8 | 81.3 | 93.3 |
| Fecal N less metabolic N (Gm.) | 0.5 | 0.43 | 0.69 | 0.62 | 1.15 |
| Absorbed nitrogen (total diet) | 4.03 | 4.22 | 3.58 | 3.72 | 13.55 |
| Digestibility of total diet protein (per cent total N absorbed) | 89.0 | 90.8 | 83.8 | 85.8 | 92.2 |

0.855 on Diet IC. These represent 12.4, 12.5, and 12.3 per cent, respectively, of the nitrogen ingested. Subtracting this excess excretion from 100 gives a digestibility of approximately 87.5 per cent.

If it be assumed that the metabolic nitrogen of all the subjects is approximately the same as for the subjects who supplied the data in Fig. 10, it is possible to estimate the digestibility of the various diets and supplements. These calculations are shown in Table IV. It is estimated that 3.00 Gm. of the total nitrogen in the diet were supplied by Diet IB. This, according to Fig. 10, would produce 0.77 Gm. of fecal nitrogen.* The difference between this figure and the total fecal nitrogen represents the fecal nitrogen or un-

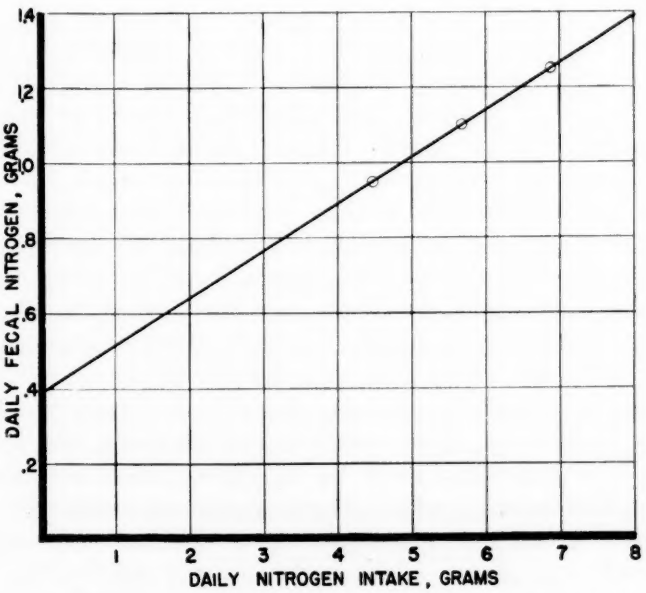


Fig. 10.—Fecal nitrogen versus nitrogen intake on all-vegetable diets—Diets IA, IB, and IC.

*This figure may be somewhat too low, since on Diets IIB, IID, and IIE the caloric supplements were increased.

digested nitrogen from the supplement, and thus the per cent of the supplement digested can be calculated.

Similarly, if the metabolic nitrogen be subtracted from the total fecal nitrogen, the fecal nitrogen from the diet is obtained. Subtracting this from the ingested nitrogen gives the dietary nitrogen absorbed. The figures obtained (Table IV) show no great differences in the digestibility of the various diets. As might be expected, the diets with meat and bread are slightly more digestible than the basic diet, while those containing soy flour and wheat germ are somewhat less. On the other hand, there appear to be marked differences in the digestibility of the supplements. Meat and bread appear highly digestible, while soy flour and wheat germ are considerably less so. Digestibility figures as determined with rats are: beef round, 96; bread, 100 (calculated from data of Mitchell and Carman[16]); and soy flour, 85.[17]

TABLE V. EFFECT OF PROTEIN SUPPLEMENTS ON BIOLOGIC VALUE OF AN ALL-VEGETABLE DIET

| | DIET | | | | |
|---|---|---|---|---|---|
| | IB | IIB | IIC | IID | IIE |
| Supplement | None | Meat | Bread | Soy flour | Wheat germ |
| Absorbed N (Gm.) | 4.11 | 4.03 | 4.22 | 3.58 | 3.72 |
| Urinary N (Gm.) | 4.29 | 3.87 | 4.35 | 4.06 | 3.43 |
| Basal calories | 1580 | 1541 | 1475 | 1504 | 1504 |
| Calculated endogenous N (Gm.) | 3.16 | 3.08 | 2.95 | 3.01 | 3.01 |
| Absorbed N excreted (Gm.) | 1.13 | 0.79 | 1.27 | 1.05 | 0.42 |
| Absorbed N retained (Gm.) | 2.98 | 3.24 | 2.95 | 2.53 | 3.30 |
| Biologic value $\frac{\text{(Retained N)}}{\text{(Absorbed N)}}$ | 72.5 | 80.4 | 70.0 | 70.6 | 88.8 |

*Estimated Biologic Value.*—Using the above determined figures for absorbed nitrogen, an estimated biologic value was calculated. For the calculation of biologic value, a figure for endogenous nitrogen (the urinary nitrogen on a nitrogen-free diet) is needed. We have used a figure of 2 mg. of nitrogen per basal calorie per day based on the data of Smuts.[12] The basal caloric requirement used is a weighted average for the subjects, assuming 37 calories per square meter per hour for women and 39.5 calories per square meter per hour for men. The calculations are shown in Table V. These figures are presented primarily for comparison since they are based on an assumed figure, and the subjects were not completely depleted. However, the figures are probably minimum biologic values and again indicate no change in the nutritional value of the diet through the addition of soybeans or more bread, while meat and wheat germ cause an improvement in biologic value. As mentioned previously, the data with wheat germ are too few to indicate the significance of the results with this supplement. The improvement in biologic value of the low protein all-vegetable diet by replacing one-third of the protein with meat is highly significant. Since biologic values are not additive, figures from the literature for separate proteins cannot be used to calculate values for mixed diets, nor can estimates of the biologic value of the individual supplements be made from data on mixed diets.

*Relation of Fecal Nitrogen to Body Weight, Caloric Intake, and Expulsion Time.*—The data available from these studies provide some information as to the causes of differences in fecal nitrogen on different diets and among individuals. Mitchell[18, 19] showed with rats that the fecal nitrogen was dependent upon the amount of food consumed. It is apparent from Fig. 10 that this is also true in these studies, and this must of course be true if digestibility for any given diet is constant at different levels of intake. On the other hand, Reifenstein and associates[20] conclude that fecal nitrogen is essentially constant over a wide range of protein intakes. These authors point out that the custom in some laboratories to assume that fecal nitrogen represents about 10 per cent of the intake gives figures much too high on high protein diets. These authors believe that the fecal nitrogen is more or less constant, at about 1.28 Gm. per day.

A comparison of the digestibility figures for meat (Table IV) in Diets IIB and F which contained meat in widely different amounts shows reasonably good agreement, 91.5 per cent and 93.3 per cent, respectively. The slightly better digestibility on the higher level of meat may be due to a lower intake of bulk, as the cornstarch biscuits which contained 0.6 per cent algin* were consumed in smaller amounts on this diet. It is not to be expected that fecal nitrogen would parallel nitrogen intake, since metabolic nitrogen constitutes a large and supposedly constant fraction if the digestibility of the diet is high. However, unless digestibility is dependent on nitrogen intake, an increase in fecal nitrogen must occur with increasing intakes. Mitchell[21] has pointed out that different samples of the same foodstuff may give rise to different values for fecal nitrogen, varying with the amount of indigestible carbohydrate, not only because of the bulk thus provided, but also because such constituents may protect in varying degree against enzyme action in the gut. Similarly, the method of preparation and degree of mastication are probably important. We feel, for example, that high fecal nitrogen figures obtained in at least one of our subjects may be partially related to poor mastication due to malocclusion.

The total fecal nitrogen has been plotted against body weight and against caloric intake (figures not shown), but the correlation in both was poor ($r = + 0.28$ for body weight; $r = + 0.28$ for caloric intake). On the other hand, we have calculated the correlation of fecal nitrogen to body weight for the data presented by Cahill and associates[22] and Murlin and co-workers.[23] Correlation coefficients of + 0.73 and + 0.77 were obtained, whereas practically no correlation with caloric intake was found.

One important cause for variations in fecal nitrogen was shown by correlating the time required for carbon markers to pass through the body (expulsion time) with the average amount of fecal nitrogen. In general, the shorter the expulsion time, the higher were the values obtained for fecal nitrogen. One subject had an expulsion time of only seven hours and a high fecal nitrogen of 1.7 Gm. per day, while another on the same diet had a fecal nitrogen of only 0.4 Gm. per day and an expulsion time of sixty-two hours.

*A sodium alginate preparation supplied by the Bushway Ice Cream Division, General Ice Cream Corporation, Somerville, Mass.

These subjects demonstrate extremes encountered and should provide a feeling of caution in the application of average figures for fecal nitrogen. Variations in bowel habits, and the effect of diet on bowel habits, are obviously important factors and probably account for greater variation in our data than in the figures presented by Cahill and associates[22] and Murlin and co-workers.[23]

*Condition of the Subjects.*—All of the subjects were well aware of the purpose of the studies. Some of them were interested in, and well informed on, the subject of protein deficiency. After a few meals, they formed a group highly subjective to suggestion. They were undoubtedly influenced by frequent questioning to determine changes in physical and mental condition. Many of them reported unusual fatigue and hunger in the late morning and afternoon, and many reported they needed more sleep than usual. These symptoms of postprandial hypoglycemia are suggestive of the finding of Thorn and associates[24] that meals high in carbohydrate and low in protein and fat are likely to be followed in two or three hours by hunger and fatigue. However, there is no doubt that those affected most adversely were those who most expected these results. The more stolid individuals and three subjects who participated in two different studies and were well accustomed to the routine were not at all sure that the low protein diet was detrimental in any way. Although a questioning of the subjects thus indicated some physical deterioration, the significance of this is doubtful.

Physical examinations, usually at weekly intervals, failed to reveal any ill effects of the diets, and body weight was maintained. Blood samples were taken at these times (except in Experiments 1 and 5) and analyses for hemoglobin, hematocrit, total plasma protein, albumin, and globulin were made, the latter three determinations by the Kjeldahl method. The results of these analyses in Experiments 2, 3, and 4 are presented in Fig. 11.

The significance of variations in different periods have been compared using the "t" test. In Experiment 2, the significant changes are a fall in serum albumin and total protein during the second period and a consistent rise in serum globulin during the protein supplemented periods. Similarly in Experiment 4, there was no significant change during the first nine days on the basic diet but a marked fall in albumin and rise in globulin during the next seven days when the subjects were on the low protein diet, in which one-third of the protein was furnished by meat. During the last ten days of the experiment on the high protein, high meat diet, there was an increase in plasma albumin and total plasma protein and a decrease in globulin. In Experiment 3, in which the low protein all-vegetable diet was continued for nineteen days, both the albumin and globulin decreased. The results of the blood analyses in these three experiments are quite consistent. In general, they show a decrease in albumin during the second week of the low protein period regardless of whether the protein source is all vegetable or one-third of it comes from meat. It is of interest to note the rise in globulin on the low protein diet containing meat (Diet IIB) compared with the fall on the basic diet in Experiment

3. Is it possible that the quality of the protein in the diet as well as the quantity has a directing influence upon the type of blood proteins synthesized and that on a diet in which the protein is reduced to near the absolute requirement, the diet containing meat favors globulin synthesis? At a still lower level of nitrogen intake (Diet G, Experiment 4), the globulins did fall while the subjects were on the meat supplemented diet. During the following period on the high meat diet, the globulin continued to fall toward a normal level as the albumin increased. The fall in albumin during the second period in Experiments 2 and 4, when one-third of the protein in the low protein all-vegetable diet was replaced by meat (Diet IIB), is not interpreted as an effect of meat supplementation, but more likely the nine days preceding, on Diet IB, were insufficient to produce the fall. This is strongly suggested by the fall in albumin over a nineteen-day period on Diet IB as shown in Experiment 3. Additional samples during this experiment might have verified this interpretation.

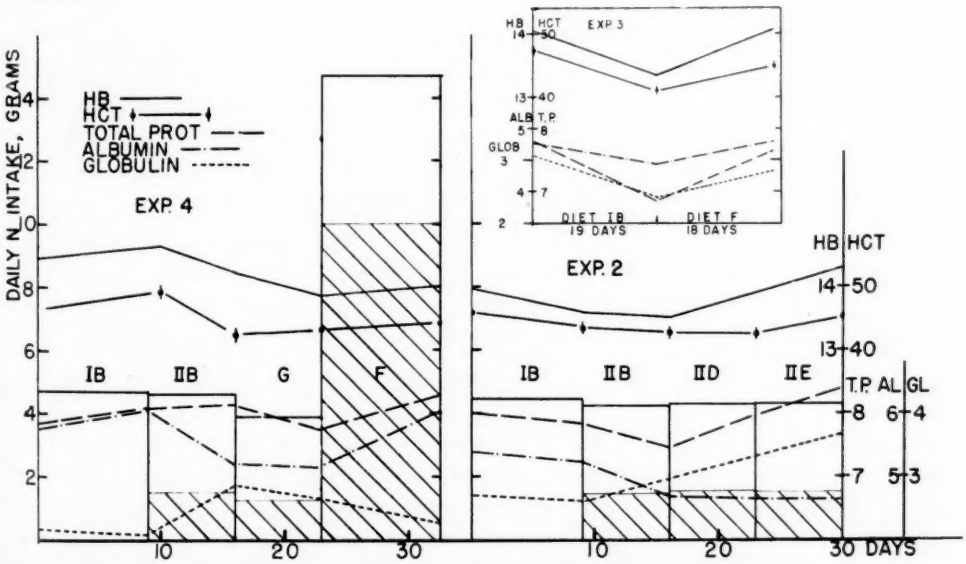


Fig. 11.—Changes in blood constituents throughout Experiments 2, 3, and 4. Areas with diagonals represent levels of nitrogen supplied by supplements.

In all experiments there was a tendency for the hematocrit to fall, which, in general, was reflected by the changes in hemoglobin concentration. Statistically, however, only the decrease during Experiment 3 was significant. All the blood constituents rapidly revert toward normal on the high protein diet.

Previous work on experimental protein deficiency in the rat[25] has shown that there is considerable decrease in plasma volume before there is any significant lowering of plasma protein concentration. Hence, plasma volume studies, using the Evans blue dye, were done on four of the subjects in Experiment 2 and on five of the subjects in Experiment 3. Measurements were made at the end of the low protein period and again at the end of the high

protein period. The plasma volume did not vary significantly over these periods, and the average of the nine subjects at the end of the all-vegetable low protein diet was 3,165 ml. as compared with 2,933 ml. at the end of the high protein period.

Basal metabolism and R.Q. measurements were made on all subjects in Experiment 4 at the end of the last low protein period (twenty-three days) and four days after being on the high protein high meat diet. The changes were slight and in line with what might be expected. Average figures of 34 calories per square meter body surface per hour at the end of the low protein period and 37 calories after four days on the high protein diet were obtained in the metabolism tests. The R.Q. decreased from 0.85 to 0.80 by changing to the high protein intake.

## DISCUSSION

It has been pointed out previously in this paper that estimates of protein requirements are most likely to err on the side which gives high values, principally because stabilized nitrogen outputs may not be obtained except in very long experiments. Lack of stabilization leads to negative balance figures which are too large and, in calculations on biologic value, to figures for retention which are too small. From the standpoint of practical nutrition, this is a fortunate occurrence, and estimates of protein requirements such as we have determined may be applied with little danger that they are low.

It is of interest to compare the protein requirement as experimentally determined in these studies with those calculated from biologic values and digestibilities. The requirement, using 2 mg. of nitrogen (0.0125 Gm. conventional protein) per basal calorie as the endogenous metabolism, may be written:

$$\text{Protein requirement in Gm.} = \frac{0.0125 \times \text{basal calories} \times 10{,}000}{\text{Biologic value} \times \text{digestibility}} + \text{Metabolic N} \times 6.25$$

The metabolic nitrogen appears to be small, probably in the range of 0.4 Gm., and equivalent to approximately 2.5 Gm. of protein. Using the figures for basal calories, digestibility, and biologic value from Tables IV and V, the calculated requirements for our subjects compared with those actually determined are given in Table VI. Reasonable agreement is obtained. The fact that the calculated requirements are in most instances somewhat high may indicate, as suggested previously, that our estimates of biologic value or digestibility are low.

It seems appropriate to interject here a brief discussion of biologic values. The significance of biologic values has received extensive and impressive support from the work of Mitchell and his collaborators.[26, 27] However, the basic supposition of a constant endogenous nitrogen metabolism appears to have been neither proved nor disproved. The findings of Schoenheimer[28] necessitate a new interpretation of Folin's original concept but do not appear to negate the possibility of a constant endogenous nitrogen excretion.[27] Recently, however, Miller[29] has shown, under conditions approximating those used for the determination of endogenous nitrogen excretion, that methionine or cystine

TABLE VI. PROTEIN REQUIREMENT IN GRAMS: COMPARISON OF VALUES CALCULATED FROM BIOLOGIC VALUE AND DIGESTIBILITY WITH VALUES DETERMINED EXPERIMENTALLY

| DIET | SUPPLEMENT | BIOLOGIC VALUE | DIGESTIBILITY | | CALCULATED REQUIREMENT (GM.) | EXPERIMENTALLY DETERMINED REQUIREMENT (GM.) |
|---|---|---|---|---|---|---|
| | | | PER CENT | NET PROTEIN VALUE | | |
| IB | None | 72.5 | 87.5 | 63.5 | 33.6 | 31.2 (Based on Fig. 6) |
| IIB | Meat | 80.4 | 89.0 | 71.5 | 29.4 | 25.4 (Based on Fig. 9) |
| IIC | Bread | 70.0 | 90.8 | 63.5 | 31.6 | 31.2 (Based on Fig. 6) |
| IID | Soy flour | 70.6 | 83.8 | 59.2 | 34.3 | 31.2 (Based on Fig. 6) |
| IIE | Wheat germ | 88.8 | 85.8 | 76.2 | 27.2 | 25.4 (Based on Fig. 9) |

supplements cause a striking fall in the urinary nitrogen output. One may calculate from his figures a biologic value of methionine alone well over 100. The dogs used in the experiment, however, were not at an endogenous level of nitrogen excretion when the methionine was fed. Confirmation of these results using certain proteins has been obtained by Swanson and co-workers[30] with rats as the experimental animal. This group of workers has used depletion periods which were more prolonged than those usually used in the determination of biologic values, and their animals are thought to be approximately at an endogenous level of metabolism. Complete evaluation of these results does not appear possible at this time. They do, however, throw doubt on many of the figures for biologic value recorded in the literature; they may indicate that the basic assumption of a constant endogenous nitrogen excretion is untrue. For the present, we prefer to retain biologic value as an exceedingly useful concept in protein studies.

It should be realized that the difference in nutritional value of the high quality and low quality proteins is simply a function of the digestibility and biologic value. It appears to us that modern nutrition instruction places a great deal of emphasis on biologic value but little on digestibility. Most textbooks of nutrition present extensive tables of biologic values without presenting the corresponding digestibility. Both values are equally important in determining the nutritional value of the protein to the individual. Perhaps the best method of comparing the relative nutritional values is to compute the product of the digestibility and biologic value. This figure, called the "net protein value," should represent the per cent of the particular protein actually available to the animal for purposes other than energy. Net protein values for the different diets used in this study are shown in Table VI.

Provided sufficient protein is fed to cover the total body requirement, considering the digestibility and biologic value, any protein source should be satisfactory. These considerations should be valid not only for maintenance in adult nutrition, but also for growth, pregnancy, and wound healing. However, in the latter states there is reason to believe that biologic values may be different and that the relative differences in the nutritional value of various proteins as measured by conventional biologic values and digestibilities may not be the same when there are special demands for protein. Mitchell[18, 19] finds, for example, lower biologic values as the level of protein in the diet is in-

creased, even though the higher levels were much below optimum for the growing rats used. This indicates a relative inefficient use of proteins for growth. Similar results are obtained in calculating the amount of protein consumed per unit gain of body weight; that is, as the amount of protein is increased, the efficiency decreases.[31] Studies on amino acid requirements[32] also emphasize the inefficiency of amino acid utilization in growth. Whether a similar situation holds for human beings, and in pregnancy and lactation, remains to be studied. Furthermore, it is well known with the rat that it is often impossible to supply sufficient protein for growth from all-vegetable diets. Whereas from 3 to 4 per cent of protein suffices for maintenance, from 15 to 20 per cent is usually required for optimum growth. Foods of high protein content are essential under these conditions.

For adults, however, we may consider some of the lower figures for digestibility and biologic values which have been reported for practical diets. There appears to be evidence that certain diets of the Far East are relatively indigestible. McCay[33] reported digestibilities of 50 per cent, but these diets contained over 700 Gm. of dry rice and a total protein content of 93 Gm. Aron and Hoscon,[34] Mason,[35] and others have also reported digestibilities between 50 per cent and 90 per cent. More recently Basu and co-workers[36, 37] have reported digestibility figures of 62, 78, 80, and 89.5 and corresponding biologic values of 75, 66.5, 60, and 59 for several Indian diets. Calculated according to the previous equation, using an average basal caloric expenditure of 1,500 calories, the protein requirement on these four diets would be 42.5, 38.7, 41.6, and 38.0 Gm. per day. It is interesting that the lower digestibility figures given are associated with higher biologic values and vice versa. We have occasionally noted similar results in experimental animals and it is probably related to the effect of differences in intake on the biologic value. For absolute comparison, proteins should be fed at equal levels of digestible protein and also at levels approximating the requirement, since it is here that the biologic values become important.

In calculating requirements from nitrogen balance studies, no consideration is given to the nitrogen loss in sweat, growth of hair, etc. At present, there appears to be insufficient data to evaluate the magnitude of these losses. Furthermore, the value of different proteins in meeting these requirements is unknown. Data obtained on chicks[38] suggest that the amino acid requirements for feather growth are considerably different from those for body growth, since birds which feather rapidly have higher arginine and glycine requirements. Poor proteins, as they are generally measured, may meet these requirements. Some allowance must undoubtedly be made above equilibrium requirements in adults, but we believe it to be small and at the present time cannot be estimated in terms of different proteins.

Protein deficiency is known to occur in adults, but it seems likely that this develops in individuals consuming natural foods, only coincident with caloric restriction. Caloric restriction over some time produces a fall in basal metabolism, blood pressure, cardiac rate, and respiratory activity. Whether

this results in a lower endogenous nitrogen and smaller protein requirements has been inadequately investigated. However, it appears logical that protein restriction and starvation produce identical effects regarding protein metabolism. In either case, tissue proteins are destroyed; in the first to meet endogenous requirements and in the second to meet caloric requirements.

No data appear available to indicate the relative value of different proteins in starvation, or indeed whether protein per se is of any value under such conditions. Out of thousands of starved individuals, both children and adults, seen in Europe immediately after the recent war, clinical and laboratory evidence of protein deficiency was found only in those who had consumed grossly insufficient amounts of calories over relatively long periods of time (three months or longer). Ninety-five to 100 per cent of the dietary protein of these starved individuals was furnished by cereal grains and potatoes, and those showing signs of protein deficiency improved simply by consuming more of these foods. Marked variations in the clinical condition of these starved patients with regard to the development of edema suggest a practical problem for research. Starvation edema involves far more than a deficiency of protein—body activity, capillary permeability, efficiency of the circulatory system, blood volume, and other physiologic factors, in addition to calories, and possibly other nutrients are also implicated.

## SUMMARY

1. Nitrogen balance studies on twenty-six adults in apparent good health were made using a low protein diet, devoid of animal protein, in which approximately 50 per cent of the protein was supplied by white bread, 12 per cent by other cereals, 30 per cent by vegetables, and 8 per cent by fruit. Adequate calories to maintain caloric equilibrium were supplied by sugars, starch, and fats. It was found that the nitrogen requirement is more closely related to surface area (basal caloric expenditure) than to body weight. The requirement for maintaining nitrogen balance was approximately 2.9 Gm. of nitrogen (18 Gm. conventional protein) per square meter of body surface. A man weighing 70 kilograms would thus require between 30 and 40 Gm. of protein, depending upon height.

2. On the same diet with one-third of the protein replaced by meat, the requirement appears to be about 2.4 Gm. nitrogen (15 Gm. conventional protein) per square meter. Protein requirement is thus about 17 per cent less on this diet than on the all-vegetable diet.

3. Data on digestibility and biologic value were determined and the equal importance of these two measurements for evaluating the nutritional value of a protein is emphasized. High quality proteins are more efficient, but less efficient proteins may serve equally well, provided enough can be fed to cover the requirement considering the specific digestibility and biologic value.

4. The biologic value of the low protein all-vegetable diet used in these studies was increased from 72.5 to 80.4 by replacing one-third of the protein with meat. The digestibility of the two diets was essentially the same.

5. There was no measurable objective change in the physical condition of any of the individuals throughout these studies. Some complained of undue postprandial hunger and fatigue on the low protein all-vegetable diets. These complaints were not present on high protein diets.

6. There were no significant changes in hemoglobin, hematocrit, or plasma volume. Total protein, plasma albumin, and plasma globulin tended to decrease on the low protein all-vegetable diet when fed at a level low enough to produce negative nitrogen balance. The replacement of one-third of the protein in this diet with meat resulted in a prompt increase in the globulin fraction.

7. It is most unlikely that protein deficiency will develop in apparently healthy adults on a diet in which cereals and vegetables supply adequate calories.

8. Considering the experimental data presented in this study, the National Research Council's daily recommended allowance of 70 Gm. of protein for an adult weighing 70 kilograms is most generous and could, if necessary, be reduced to 50 Gm. and still provide approximately 30 per cent margin above requirement.

9. It should be emphasized that the experimental data and the conclusions of this paper apply to adults in apparent good health and do not consider protein requirements in growth, pregnancy, lactation, or disease.

## ADDENDUM

Since this paper was written, an important contribution on the protein requirements of adults* has appeared. It is interesting that these authors' data upon a mixed diet which corresponds in some respects to our diet containing meat yield values for the protein requirements similar to those obtained in this study. These authors have calculated protein requirements to include nitrogen losses in sweat, skin, and hair. As discussed previously we believe these requirements have been too little studied to be evaluated at this time, especially in terms of specific proteins.

It is also of interest that these authors find the endogenous nitrogen losses, although determined on only two subjects, to be approximately 1.4 mg. per basal calorie. This figure corresponds closely to that found by Ashworth and Brody on rats rather than the figure of 2 mg. given by Smuts.

We are indebted to Simmons College and particularly to Dr. E. Robb, Professor of Home Economics, for the use of kitchen facilities required in these studies, and to the American Meat Institute, for a grant-in-aid which made this work possible.

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# PLASMA PROTEIN LEVELS IN NORMAL INDIVIDUALS

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DURHAM, N. C.

MORE than 1,500 semi-micro-Kjeldahl determinations of total plasma proteins and albumin in as many different individuals were part of a nutrition survey carried out over a period of five and one-half years in North Carolina (1940-1945). It seemed of value, therefore, to present these extensive data in a series of frequency distributions of persons grouped by race, sex, and age.

The technique used and the characteristics of areas and groups of persons surveyed have been described in previous communications.[1-3] It is noteworthy that the entire study pertained to individuals in families who were presumably in ordinary health, living in their customary environment and at their usual occupations. Their nutritional status was that prevailing in this region at the time, and they exhibited no signs of protein malnutrition. The mean intake of protein by adults was from 60 to 90 Gm. daily, varying with year and area of survey. That of men was usually above 80 Gm.

The available data on blood protein levels in presumably normal individuals are not extensive. Peters and Van Slyke[4] tabulated the normal values for sixteen women and sixteen men. Peters and Eisenman[5] reported values for fifty-two "normal" persons and found 90 per cent of the levels of total proteins between the limits of 6.3 and 7.7 Gm. per cent. Myers and Muntwyler,[6] in reviewing the literature to 1940, found normal levels to be from 5.6 to 8.4 Gm. for total proteins, from 3.4 to 5.6 Gm. for albumin, and from 1.35 to 3.55 Gm. for globulin, giving A/G ratios of 1.2 to 2.6. Kagan,[7] reporting on 150 normal persons tested by the falling drop method, found serum protein levels of from 6.1 to 7.6 Gm. (average, 6.7 Gm.) and considered values greater than 7.5 Gm. or less than 6.0 Gm. as abnormal until proved otherwise. Youmans and co-workers[8] reported protein levels on a survey of 776 individuals in Tennessee and found the mean level to be 6.95 ± 0.02 Gm. per cent. These levels were based only partly on Kjeldahl determinations, since a large number were obtained by a biuret technique. Robinson and associates[9] reported a survey on 439 normal subjects in Mexico City (altitude, 8,500 feet) having a mean protein level of 7.75 ± 0.03 Gm. per cent. This high mean must be evaluated in the light of the population surveyed and of its location, and also in the light of the fact that determinations were based entirely on the biuret method.

The present study pertains to by far the largest group of subjects examined to date. The semi-micro-Kjeldahl technique was used throughout. The 1,561 determinations were made on as many different individuals. Since oxalated plasma was used, results are not strictly comparable with those of surveys in which serum was employed.

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The surveys reported here were carried out under the auspices of North Carolina State Board of Health, the International Health Division of The Rockefeller Foundation, and Duke University School of Medicine.

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## METHODS

Blood collections were made during the period of from 9 to 12 A.M.; there was no request that individuals omit breakfast. A tourniquet was applied and 25 c.c. of blood rapidly withdrawn into a syringe and emptied into a bottle containing dried oxalate (0.1 c.c. of 25 per cent potassium oxalate dried in the oven for from fifteen to twenty minutes). The amount of blood actually withdrawn usually approximated 25 c.c. with a small percentage of unavoidable variations.

Determinations of total protein and albumin were made by a semi-micro-Kjeldahl procedure. The ammonia produced by wet-ashing the samples with a selenium copper sulfate sulfuric acid digestion mixture and superoxal was liberated with alkali and distilled into 10 c.c. of 2 per cent boric acid. Using a methyl red methylene blue indicator, the ammonia was titrated with accurately standardized 0.02 M HCl. For total protein, a sample of 0.2 c.c. of plasma measured with a micro-pipette was digested directly with 3 c.c. of digestion mixture; for albumin, 0.5 c.c. of plasma was precipitated with 14.5 c.c. of 22.5 per cent sodium sulfate solution and allowed to settle overnight at 37° C., and 10 c.c. of a clear filtrate were taken for digestion with the same amount of digestion mixture. Both determinations were carried out at least in duplicate, and only titrations checking within 0.20 c.c. 0.02 N acid were accepted.

## DISCUSSION

In Table I are presented the mean values for total proteins and for albumin and globulin separately for eight subdivisions of the surveyed population by age, sex, and color. Since differences in mean levels with respect to age and sex were obviously minor, further analysis was confined to the results obtained for white and colored populations specifically. In Figs. 1 and 2 are presented the observed frequency distributions of the persons in these two population groups according to total proteins, albumin, and globulin.

It is apparent that the frequency distributions are essentially "normal" in type; hence Figs. 1 and 2 contain normal frequency curves fitted to the ob-

TABLE I. PLASMA TOTAL PROTEINS, ALBUMIN, AND GLOBULIN MEAN LEVELS FOR RACE, SEX, AND AGE GROUPS

| AGE AND SEX GROUPS | NUMBER IN GROUP | | GRAMS PER CENT | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | TOTAL PROTEINS | | ALBUMIN | | GLOBULIN | | A/G RATIO | |
| | WHITE | COLORED | WHITE | COLORED | WHITE | COLORED | WHITE | COLORED | WHITE | COLORED |
| Under 15 yr. | | | | | | | | | | |
| Male | 224 | 114 | 7.10 | 7.37 | 4.65 | 4.49 | 2.49 | 2.93 | 1.87 | 1.53 |
| Female | 257 | 113 | 7.21 | 7.49 | 4.78 | 4.56 | 2.49 | 2.99 | 1.92 | 1.53 |
| Total | 481 | 227 | 7.16 | 7.43 | 4.72 | 4.52 | 2.49 | 2.96 | 1.90 | 1.53 |
| 15† yr. | | | | | | | | | | |
| Male | 267 | 82 | 7.12 | 7.39 | 4.59 | 4.39 | 2.58 | 3.05 | 1.78 | 1.44 |
| Female | 387 | 117 | 7.29 | 7.50 | 4.61 | 4.37 | 2.73 | 3.18 | 1.69 | 1.37 |
| Total | 654 | 199 | 7.22 | 7.46 | 4.60 | 4.38 | 2.67 | 3.13 | 1.72 | 1.40 |
| Grand total* | 1135† | 426† | 7.19 | 7.44 | 4.65 | 4.45 | 2.59 | 3.04 | 1.80 | 1.46 |

*Difference of 0.05 in means from Table II due to difference of definition of group centers.

†Albumin determinations totaled 1,098 white, 423 colored; globulin, 1,094 white and 423 colored.

served data, which are represented by histograms.[10] These fitted curves describe the distributions quite well, with the differences due less to systematic shifts than to apparently irrational discrepancies in the number of persons observed in occasional subgroups near the middle of the range of protein values.

When a frequency distribution may be legitimately described by a normal curve, one may easily proceed to calculate the probability that the protein level of one or more persons may fall between any two given limits by using the mean and the standard deviation (sigma) of the curve. On the base scale of each figure are entered the position and the numerical value of the mean as well as the location and values of points exceeding or falling below this value by one, two, and three times the standard deviation.

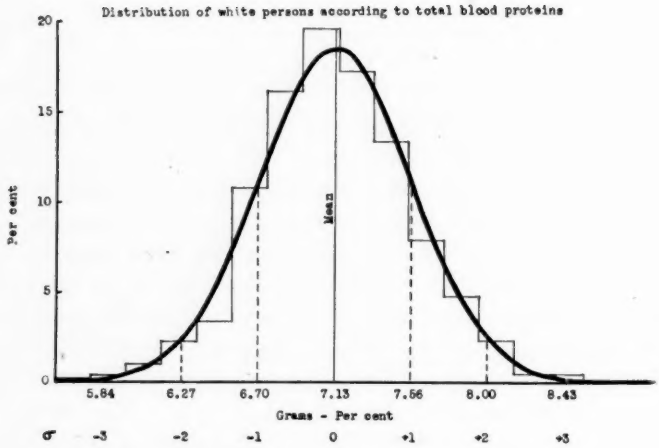
To facilitate comparisons, the means and standard deviations for each group, together with their standard errors, are collected in Table II. In addition there are tabulated values between which one-half the observations drawn from a "normal" population may be expected to lie (on the basis of the fitted curve) and another pair of values which would be expected to include nineteen-twentieths of all observations. Persons with protein levels falling accidentally outside these wider limits would rarely be found. Conversely, when persons with such values are encountered they should be strongly suspected as having some pathologic condition.

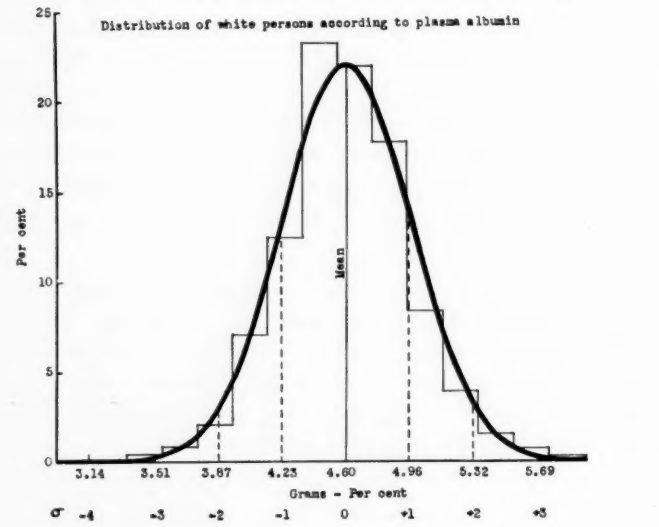
The means in Table II indicate that colored persons possessed a significantly higher level of total proteins and globulin than did white persons, while the albumin level was higher among white persons. The actual differences between the levels of the means, however, were not great in view of the scatter of the observed values as indicated by the standard deviations. The A/G ratio was distinctly higher in the white population (1.80) than in the colored (1.46).

Precipitin tests for syphilis were made on each individual in the survey. The incidence of positive results was low—less than 3 per cent in colored and 1 per cent in white persons. Malaria was not endemic in three of the four

TABLE II. DISTRIBUTION CONSTANTS OF PLASMA TOTAL PROTEINS, ALBUMIN, AND GLOBULIN FOR WHITE AND COLORED POPULATIONS, WITH LIMITS BETWEEN WHICH ONE-HALF AND NINETEEN-TWENTIETHS OF THE OBSERVATIONS MAY BE EXPECTED TO FALL

| | TOTAL PROTEINS | ALBUMIN | GLOBULIN |
|---|---|---|---|
| Mean | | | |
| White | 7.13 ± 0.013 | 4.60 ± 0.011 | 2.54 ± 0.013 |
| Colored | 7.38 ± 0.023 | 4.40 ± 0.016 | 2.99 ± 0.021 |
| Difference and standard error | 0.25 ± 0.026 | 0.20 ± 0.019 | 0.45 ± 0.025 |
| Standard deviation | | | |
| White | 0.430 ± 0.009 | 0.363 ± 0.008 | 0.422 ± 0.009 |
| Colored | 0.469 ± 0.016 | 0.320 ± 0.011 | 0.429 ± 0.015 |
| Limits of one-half of distribution | | | |
| White | 6.84 - 7.42 | 4.35 - 4.84 | 2.25 - 2.82 |
| Colored | 7.07 - 7.70 | 4.18 - 4.62 | 2.70 - 3.28 |
| Limits of nineteen-twentieths of distribution | | | |
| White | 6.29 - 7.98 | 3.89 - 5.31 | 1.71 - 3.37 |
| Colored | 6.46 - 8.30 | 3.77 - 5.03 | 2.15 - 3.83 |


Distribution of white persons according to total blood proteins
Per cent
20
15
10
5
0
Mean
5.84
6.27
6.70
7.13
7.56
8.00
8.43
Grams - Per cent
σ -3 -2 -1 0 +1 +2 +3


Distribution of white persons according to plasma albumin
Per cent
25
20
15
10
5
0
Mean
3.14
3.51
3.87
4.23
4.60
4.96
5.32
5.69
Grams - Per cent
σ -4 -3 -2 -1 0 +1 +2 +3

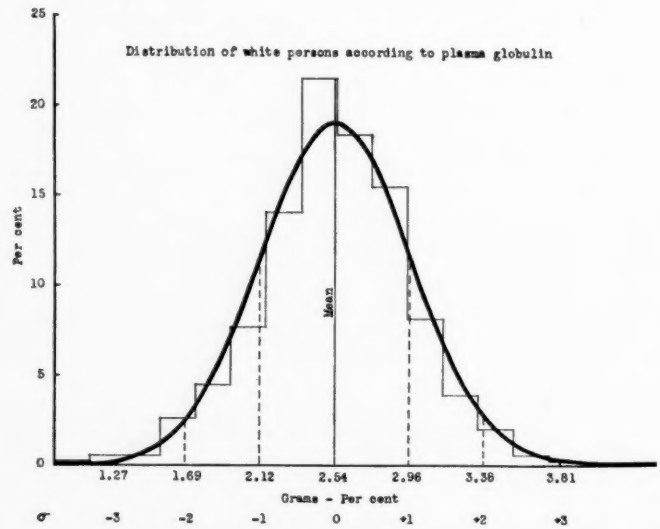
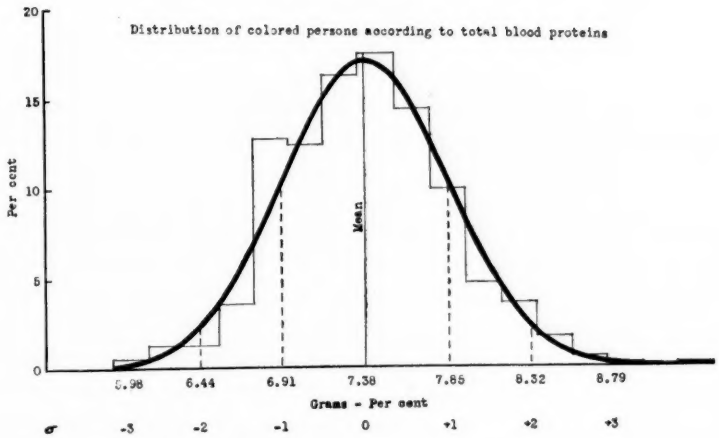
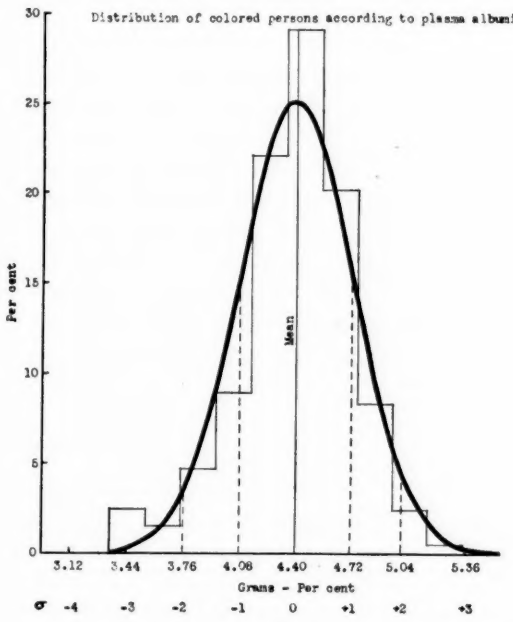

Distribution of white persons according to plasma globulin
Per cent
25
20
15
10
5
0
Mean
1.27
1.69
2.12
2.54
2.96
3.38
3.81
Grams - Per cent
σ -3 -2 -1 0 +1 +2 +3

Fig. 1.


Distribution of colored persons according to total blood proteins
Per cent
Mean
5.98
6.44
6.91
7.38
7.85
8.32
8.79
Grams - Per cent
σ -3 -2 -1 0 +1 +2 +3


Distribution of colored persons according to plasma albumin
Per cent
Mean
3.12
3.44
3.76
4.08
4.40
4.72
5.04
5.36
Grams - Per cent
σ -4 -3 -2 -1 0 +1 +2 +3

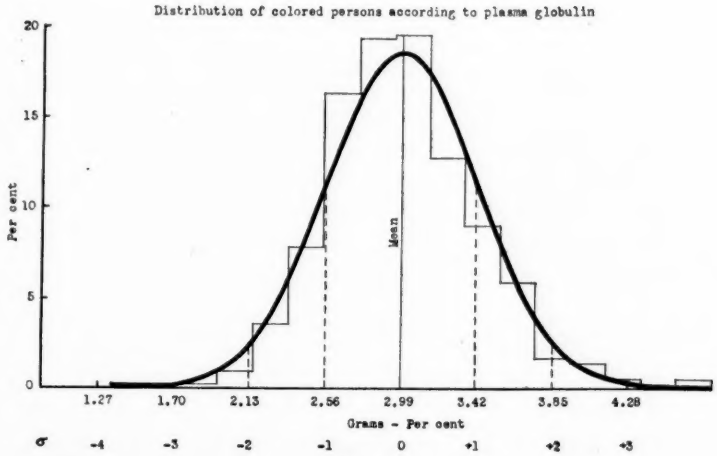

Distribution of colored persons according to plasma globulin
Per cent
Mean
1.27
1.70
2.13
2.56
2.99
3.42
3.85
4.28
Grams - Per cent
σ -4 -3 -2 -1 0 +1 +2 +3

Fig. 2.

counties surveyed. In the other, only one positive (thick) blood smear was found in nearly 900 examinations.

## SUMMARY

This paper presents an analysis of blood protein determinations on 1,561 individuals, performed on oxalated plasma by the semi-micro-Kjeldahl method. The individuals, all presumably in normal health, lived on or near the coastal plain in North Carolina.

Blood protein levels of white persons differed somewhat from those of colored individuals, but the factors of age and sex could be disregarded. "Normal" curves well described the distributions of persons in each racial group based on total proteins, or on albumin and globulin separately. The fitted curves are presented in Figs. 1 and 2 and their constants given.

The mean total protein level for white persons was 7.19 Gm. per cent and for colored persons, 7.44. The mean albumin was higher among white than among colored individuals (4.65 and 4.45 Gm. per cent, respectively). The globulin level was, therefore, higher in the colored group (3.04 versus 2.59 Gm. per cent). Mean A/G ratios were 1.80 among white and 1.46 among colored persons. The difference between the races in globulin levels was twice as great as that between age and sex groups of the same race.

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# THE TREATMENT OF BACTERIAL ENDOCARDITIS WITH PENICILLIN

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ALTHOUGH the efficacy of penicillin in the treatment of some bacterial infections is well established, its effect in bacterial endocarditis has been more difficult to evaluate. Herewith are presented the results in twenty-eight patients with streptococcal and staphylococcal endocarditis treated with penicillin* in the three-year period from December, 1942, to December, 1945. This report represents a continuation of the cooperative clinical study of the therapeutic effect of penicillin in bacterial infections and includes further observations of three patients previously reported.[1]

## METHODS

*Selection of Patients.*†—In this series, all patients with adequate clinical and laboratory evidence of bacterial endocarditis were included, regardless of the patient's condition at the time of admission to the hospital.

*Administration of Penicillin.*—Although intermittent and continuous methods were used either intravenously or intramuscularly on various occasions, the usual procedure was the intramuscular injection of 40,000 units every two hours day and night. The use of a continuous intramuscular drip was abandoned because of reactions.[2] Continuous intravenous infusion was employed for the most part only when it was necessary to give massive doses. When bacteremia persisted after several days of therapy, the dose of penicillin was increased.

The duration of treatment was variable, but in the more recent cases it was continued until the patient was afebrile for at least six weeks.

*Other Therapy.*—Sulfonamide drugs were given to two patients, to one because the infecting organism was resistant to penicillin, and to the other because of a concomitant fungus infection. Anticoagulants were used once and then only in an attempt to maintain a continuous intravenous drip. Supportive measures such as blood transfusions, high calorie diets, and supplementary vitamins were employed freely as indicated.

*Observations.*—Special efforts were made to maintain complete and accurate records on each patient throughout his stay in the hospital.

With one exception (Case 7), two or more cultures of the blood were positive before the beginning of treatment, and, in that instance, the clinical manifestations were so striking as to justify the diagnosis. In several critically ill

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*The penicillin was provided by the Office of Scientific Research and Development from supplies assigned by the Committee on Medical Research for clinical investigations recommended by the Committee on Chemotherapeutic and Other Agents of the National Research Council.

†Of the twenty-eight patients comprising this report, twenty-five were treated in the Barnes Hospital and three in the St. Louis City Hospital.

TABLE I. TREATMENT OF STREPTO

| CASE | AGE | SEX | TYPE OF HEART DISEASE | DURATION OF SYMPTOMS | MURMURS* | NUMBER OF POSITIVE BLOOD CULTURES BEFORE TREATMENT | MAXIMUM COLONY COUNTS PER C.C. | ORGANISM |
|---|---|---|---|---|---|---|---|---|
| 1 | 68 | Female | Rheumatic | 5 mo. | M.I. | 6 | 200 | Streptococcus viridans |
| 2 | 24 | Female | Congenital | 1 mo. | P.S., P.I. | 7 | 120 | Streptococcus viridans |
| 3 | 25 | Female | Rheumatic | 6 mo. | A.S., A.I., M.S. | 5 | 320 | Streptococcus viridans |
| 4 | 56 | Male | Rheumatic | 1 mo. | M.S., A.I., M.I. | 4 | | Streptococcus viridans |
| 5 | 38 | Male | Rheumatic syphilitic | 1½ mo. | A.I., A.S. | 2 | | Streptococcus viridans |
| 6 | 22 | Male | Rheumatic | 2 mo. | M.S., M.I. | 6 | | Streptococcus viridans |
| 7 | 50 | Male | Rheumatic | 1 wk. | M.S., M.I., A.S., A.I. | 0‡ | | |
| 8 | 29 | Male | Rheumatic | 1 mo. | M.I., M.S. | 2 | | Streptococcus viridans |
| 9 | 49 | Male | Rheumatic | 5 mo. | M.I. | 4 | | Streptococcus viridans |
| 10 | 24 | Female | Rheumatic | 2 mo. | M.I. | 4 | 335 | Streptococcus viridans + |
| 11 | 56 | Female | Rheumatic | 6 mo. | M.I., M.S. | 6 | | Streptococcus viridans§ |
| 12 | 62 | Male | Rheumatic | 5 mo. | M.I., A.I. | 4 | | Microaerophilic nonhemolytic streptococcus |
| 13 | 20 | Male | Rheumatic | 6 mo. | M.I., M.S. | 4 | | Streptococcus viridans |
| 14 | 55 | Male | Rheumatic | 6 mo. | M.I., M.S., A.I. | 4 | 120 | Streptococcus viridans ++ |
| 15 | 30 | Male | Rheumatic | 2 mo. | M.I. | 3 | | Streptococcus viridans |
| 16 | 37 | Female | Rheumatic | 4 mo. | M.S., M.I., A.I. | 4 | | Streptococcus viridans |
| 17 | 26 | Female | Congenital | 4 mo. | P.D.A.‖ | 4 | 34 | Streptococcus viridans |
| 18 | 32 | Female | Rheumatic | 8 mo. | M.I., M.S. | 7 | 60 | Streptococcus viridans |
| 19 | 59 | Male | Rheumatic | 6 mo. | M.I. | 4 | 100 | Streptococcus viridans |
| 20 | 41 | Male | Rheumatic | 6 mo. | M.S. | 4 | 35 | Streptococcus viridans |
| 21 | 22 | Female | Rheumatic | 10 mo. | M.I., M.S. | 4 | | Streptococcus viridans |
| 22 | 15 | Female | Congenital | 4 days | P.D.A.¶ | 6 | 182 | Streptococcus viridans |

*M.S., Mitral diastolic murmur; M.I., mitral systolic murmur; A.S., aortic systolic murmur; A.I., aortic diastolic murmur; P.S., pulmonic systolic murmur; P.I., pulmonic diastolic murmur; P.D.A., to and fro murmur of patent ductus arteriosus.

†See Appendix.

‡Many negative blood cultures including those obtained by sternal and arterial routes.

§Also *Actinomyces bovis* five times.

patients in whom the clinical findings were indicative of bacterial endocarditis, three or four blood cultures were drawn over a period of several hours, after which therapy with penicillin was instituted. In each of these patients the blood cultures were subsequently found to be positive and the diagnosis confirmed. Frequent blood cultures were obtained throughout and immediately following the course of therapy.

COCCAL ENDOCARDITIS WITH PENICILLIN

| RANGE OF DAILY DOSE (UNITS) | ROUTE | DURATION OF TREATMENT (DAYS) | TOTAL PENICILLIN (UNITS) | RESULT |
|---|---|---|---|---|
| 300,000 | I.M. | 7 | 1,700,000 | Died on the seventh day of therapy; autopsy revealed rheumatic mitral disease with active vegetations |
| 80,000-700,000 | I.M., I.V. | 73 | 13,783,500 | Well eighteen months after therapy |
| 120,000-720,000 | I.M. | 52 | 17,580,000 | Well nineteen months after therapy† |
| 240,000 | I.M. | 25 | 5,980,000 | Died one month after therapy† |
| 480,000 | I.M. | 3 | 960,000 | Died on third day of therapy; autopsy showed aortic and mitral rheumatic valvulitis with active vegetations, bicuspid aortic valve, syphilitic aortitis |
| 480,000 | I.M. | 19 | 5,850,000 | Well fourteen months after therapy† |
| 240,000-1,200,000 | I.M. | 56 | 38,600,000 | Well ten months after therapy; one episode of cardiac decompensation |
| 240,000-500,000 | I.M. | 60 | 23,300,000 | Well ten months after therapy |
| 240,000 | I.M. | 3 | 580,000 | Died on third day of therapy probably from cerebral embolus; autopsy revealed rheumatic mitral disease with active vegetations; brain not examined |
| 480,000-5,000,000 | I.M., I.V. | 49 | 167,755,000 | Alive and improved; blood cultures positive on discharge† |
| 480,000-960,000 | I.M. | 11 | 6,720,000 | Died on eleventh day of therapy; autopsy revealed mitral and tricuspid disease with active vegetations on both |
| 240,000-480,000 | I.M. | 35 | 12,060,500 | Well eight months after therapy |
| 240,000-720,000 | I.M. | 50 | 22,462,000 | Well eight months after therapy |
| 480,000-1,200,000 | I.M., I.V. | 28 | 22,310,000 | Died of cardiac insufficiency twenty-eight days after therapy; resistant organism; autopsy revealed aortic and mitral rheumatic disease with active vegetations |
| 240,000-480,000 | I.M. | 40 | 15,320,000 | Well six months after therapy |
| 240,000-480,000 | I.M. | 42 | 14,500,000 | Died one month after therapy in acute cardiac failure; no evidence clinically of recurrence; no autopsy |
| 480,000-960,000 | I.M. | 65 | 29,580,000 | Well four months after therapy |
| 480,000 | I.M. | 43 | 20,200,000 | Well four months after therapy |
| 240,000-480,000 | I.M. | 74 | 32,880,000 | Well three months after therapy |
| 480,000-960,000 | I.M. | 61 | 32,600,000 | Well three months after therapy |
| 1,000,000 | I.V. | 4 | 4,150,000 | Died on the fourth day of therapy; no autopsy† |
| 480,000 | I.M. | 68 | 32,160,000 | Well two months after therapy |

‖Ligation of patent ductus arteriosus by Dr. Evarts D. Graham during therapy.

¶Religation of patent ductus arteriosus by Dr. Karl Poppe during therapy.

+, Relatively resistant to penicillin (see Appendix).

++, Relatively resistant to penicillin; not inhibited by concentrations less than 2.5 units per cubic centimeter.

## The following technique for blood cultures was used:

Twenty-three cubic centimeters of blood were drawn with aseptic precautions and transported from the bedside to the laboratory in a flask containing 3 c.c. of a 4 per cent solution of sodium citrate. A flask containing 100 c.c. of beef infusion broth was inoculated with 10 c.c. of blood, and for growth under reduced oxygen tension 10 c.c. of blood were placed in a flask of thioglycollate broth.[3] The latter medium was contained in a Florence flask of 125 c.c. capacity in order to minimize the area of the surface in contact with the air. In our ex-

perience, growth of *Streptococcus viridans* was especially prompt and abundant in thioglycollate broth. In addition, pour plates were made with 1 and 2 c.c. amounts of blood. Each 100 c.c. of medium contained 5 mg. of para-aminobenzoic acid[4] and, during the latter part of the study, 1 mg. of cysteine hydrochloride was also added in order to inactivate penicillin carried into the culture from the blood of the patient.[5, 6] The cultures were observed daily and subcultures made when indicated.

In vitro sensitivity to penicillin* of many of the streptococcal strains was tested† with various methods, but the results of repeated experiments were not consistent enough to state reliably the number of units of penicillin needed for antibacterial action on a given strain. However, it was readily recognized and confirmed that strains from two patients were relatively resistant.

The clinical condition of all surviving patients has been evaluated frequently and information regarding fever, embolic phenomena, anorexia, weight loss, and malaise has been especially sought. Blood cultures have been obtained whenever possible. Except in two instances, post-mortem examinations have been carried out on the patients who have died.‡

## RESULTS

*Streptococcal Endocarditis.*—Twenty-two of the patients had streptococcal endocarditis and significant data concerning them are summarized in Table I. The number of survivors with penicillin therapy and the duration of the period since termination of treatment are shown in Tables II and III. Illustrative case histories are presented in the appendix. It should be noted that a complete course of therapy was given to fifteen patients, and of these, thirteen are now living and well (Appendix, Case 6). On the other hand, of the five patients who died before the completion of therapy, three died within the first four days of treatment (Cases 5, 9, and 21) and the other two within eleven days (Cases 1

TABLE II. RESULTS OF PENICILLIN TREATMENT OF PATIENTS WITH BACTERIAL ENDOCARDITIS

| ORGANISM | NUMBER OF CASES | LIVING AND WELL | FAILURES | |
|---|---|---|---|---|
| | | | LIVING BUT WITHOUT ARREST OF INFECTION | DIED |
| Streptococcal | 22 | 13 | 1 | 8 |
| Staphylococcal | 6 | 5 | 0 | 1 |
| Total | 28 | 18 | 1 | 9 |

TABLE III. LENGTH OF FOLLOW-UP IN PATIENTS NOW CLINICALLY WELL

| | |
|---|---|
| Eighteen to twenty-four months | 4 |
| Twelve to eighteen months | 1 |
| Six to twelve months | 6 |
| Three to six months | 5 |
| One to three months | 2 |
| Total | 18 |

*A standard sample of calcium penicillin was kindly furnished by Dr. Henry Welch, of the Food and Drug Administration.

†These procedures were carried out by Miss Ruth Peterson under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Washington University School of Medicine.

‡These will be reported by Dr. Robert A. Moore, of the Department of Pathology.

and 11). Two patients (Cases 2 and 3) suffered immediate relapses after twenty-four and thirty days of therapy, but both responded well to second courses. In one (Case 2), relapse occurred the day after penicillin was discontinued; in the other, fever began four days after the last dose of the drug (Appendix, Case 3). Two of the patients died in congestive heart failure (Cases 4 and 16). One of these showed no bacteria in sections of the involved heart valve obtained at autopsy (Appendix, Case 4). The other had no clinical evidence of infection at the time of death, but autopsy observations could not be made. The streptococci isolated from two patients (Cases 10 and 14) were relatively resistant to penicillin. One of these patients died, but the other is living and clinically well, although bacteria were still present in the blood at the time the patient left the hospital (Appendix, Case 10).

*Staphylococcal Endocarditis.*—The six cases of endocarditis caused by staphylococci are summarized in Table IV. An acute and fulminating course was observed in four of the patients (Cases 23, 25, 26, and 28), while a chronic afebrile disease in one (Case 27) was correlated with characteristics of the organism usually associated with lack of pathogenicity.[7, 8]

Results in this group were excellent; there was only one death and that occurred in the first patient in the entire series (Case 23). It is interesting to note that this patient made a good response to small doses of penicillin; death was due to rupture of a brain abscess.

## DISCUSSION

Prior to the use of penicillin, the prognosis of bacterial endocarditis was almost hopeless.[9-13] Although the first reports on the use of the drug in the treatment of this disease were discouraging,[14-25] many observers have subsequently reported favorable results.[26-27] Our results likewise confirm the efficacy of penicillin. The magnitude of the advance is strikingly demonstrated in Table V, in which the results in the present series are compared with those obtained in twenty-four patients with streptococcal and staphylococcal endocarditis admitted to the Barnes Hospital over a seven-year period from 1935 to 1941 inclusive.*

*Criteria of Cure.*—Observations of the natural course of bacterial endocarditis in the years before penicillin therapy have shown that spontaneous remissions occur[9] and may lead to false conclusions as to the prognosis. In view of this fact, it has been pointed out[44] that a long follow-up period is necessary in order to demonstrate the value of treatment in this disease. In the present series, it is possible to report that a considerable number of patients are alive and apparently well after periods of from six months to two years since the conclusion of treatment. These observations indicate that beneficial effect is not of a temporary character and increase the probability of actual cure.

In most patients with bacterial endocarditis in whom the infection seems to be controlled with penicillin, it is impossible to observe the microscopic lesions of the valve because the patients are living. In three reported cases[26, 49] in

*Twenty of these cases were due to *Streptococcus viridans*, three to hemolytic *Staphylococcus aureus*, and one to hemolytic *Staphylococcus albus*.

TABLE IV. TREATMENT OF STAPHYLOCOCCAL

| CASE | AGE | SEX | TYPE OF HEART DISEASE | DURATION OF SYMPTOMS | MURMURS* | NUMBER OF POSITIVE BLOOD CULTURES BEFORE TREATMENT | MAXIMUM COLONY COUNTS PER C.C. | ORGANISM |
|---|---|---|---|---|---|---|---|---|
| 23 | 33 | Female | Rheumatic | 8 days | M.I., M.S., A.I. | 6 | 1000 | Hemolytic Staphylococcus aureus‡ |
| 24 | 31 | Female | Congenital | 5 wk. | I.V.S. | 3 | 200 | Hemolytic Staphylococcus aureus‡ |
| 25 | 17 | Female | Rheumatic | 6 days | M.I. | 3 | 160 | Hemolytic Staphylococcus aureus‡ |
| 26 | 35 | Female | None | 3 wk. | M.I.‖ | 4 | | Hemolytic Staphylococcus aureus‡ |
| 27 | 35 | Male | Rheumatic | 5 mo. | M.I., M.S. | 12 | 84 | Hemolytic Staphylococcus albus§ |
| 28 | 22 | Male | None | 15 days | M.I.‖ | 4 | | Hemolytic Staphylococcus aureus‡ |

*M.I., Mitral systolic murmur; M.S., mitral diastolic murmur; A.I., aortic diastolic murmur; I.V.S., systolic murmur of patent interventricular septal defect.
†See appendix.

which the infection was apparently arrested with penicillin, the opportunity to observe sections of the involved valves presented itself when the patients subsequently died of cardiac failure. In two of these cases,[49] no bacteria were found and evidence of extensive healing was present; in the third,[26] although the reparative process was noted, a small nest of bacteria was demonstrated. In one of our patients (Case 4) death from heart failure made it possible to examine a section of the affected valve and no bacteria were seen. Inasmuch as sections of valves in untreated bacterial endocarditis have almost always shown numerous large colonies of bacteria,[9, 50-52] the foregoing observations constitute strong evidence in favor of the theory that patients with bacterial endocarditis who are clinically well for several months after penicillin treatment are completely cured.

*Analysis of Failures.*—While a significant number of patients have made an apparent recovery from bacterial endocarditis and while the value of penicillin therapy in this disease seems established, it is desirable to consider carefully those patients in whom the ultimate outcome was unsatisfactory in an attempt to evaluate the factors responsible.

The importance of prompt, intensive, and prolonged penicillin treatment is indicated by the failures recorded in six of our patients who died early in the course of therapy; it is further emphasized when one considers the progressive debility which accompanies systemic infection and the danger of disabling or fatal embolism[53] (Appendix, Case 21).

Failure in two of our patients with streptococcal endocarditis (Cases 10 and 14) was adequately explained by the relative resistance to penicillin of the bacteria isolated. It has been shown that certain strains of streptococci are inherently resistant to penicillin[54, 55]; this is particularly true of *Streptococcus fecalis*,[54, 56] which causes from 5 to 10 per cent of all cases of streptococcal endocarditis.[54] In view of these facts, it is clear that at present insensitive bacteria may interfere with recovery in a significant number of patients. In one of our

ENDOCARDITIS WITH PENICILLIN

| RANGE OF DAILY DOSE (UNITS) | ROUTE | DURATION OF TREATMENT (DAYS) | TOTAL PENICILLIN (UNITS) | RESULTS |
|---|---|---|---|---|
| 60,000 | I.V. | 5 | 270,000 | Died on fifth day of therapy; autopsy revealed rheumatic mitral disease with active vegetations and ruptured brain abscess |
| 360,000 | I.V. | 13 | 4,680,000 | Well twenty-one months after therapy |
| 40,000-300,000 | I.M., I.V. | 26 | 3,120,000 | Well twenty months after therapy† |
| 200,000-1,200,000 | I.M. | 22 | 11,600,000 | Well ten months after therapy |
| 480,000 | I.M. | 42 | 20,512,000 | Well four months after therapy; one episode of cardiac decompensation |
| 240,000-1,200,000 | I.M., I.V. | 67 | 58,640,000 | Well one and one-half months after therapy |

‡Coagulase positive, mannite fermented.
§Coagulase negative, mannite not fermented.
‖Murmur developed under observation.

patients (Appendix, Case 10) an attempt was made to give enough penicillin to overcome a resistant streptococcus, and a striking decrease in the number of bacteria in the blood was observed. The practical difficulties encountered in the administration of this large amount of penicillin rendered continuation of such therapy impossible, but the results seem encouraging enough to warrant further trials if it becomes feasible to attain sufficiently high levels of penicillin in the body.

In two of our patients (Cases 2 and 3; Appendix, Case 3) relapse occurred, apparently because penicillin was not continued long enough. The histories of several others (Cases 10, 15, 16, 17, 20, and 21) showed that penicillin had been given to them for short periods of time before they came to this hospital and that the clinical manifestations of the disease had recurred shortly after therapy was concluded. In all but one of these patients further prolonged treatment with penicillin resulted in a satisfactory arrest of the infection. In the single unsuccessful instance (Case 10), the evidence points more to inherent resistance of the organism as previously noted. The experience, as a whole, supports the opinion[26, 29, 46, 57, 58] that penicillin therapy should be continued over a long period of time and does not indicate that the bacteria are likely to acquire enough resistance through one or two inadequate courses of treatment to interfere with subsequent success.

Two patients (Cases 4 and 16) died of cardiac insufficiency and two more (Cases 7 and 27) had episodes of heart failure in spite of the fact that their infections were apparently arrested by penicillin. It has been suggested that cardiac failure may be due to valvular deformity produced by fibrous contractions of healing vegetations,[46, 49, 58] particularly if the aortic valve is involved.[34, 49, 58] Although it is not possible to prove that such a mechanism was responsible for heart failure in our patients, it seems probable that ultimate

TABLE V. RESULTS OF THE TREATMENT OF BACTERIAL ENDOCARDITIS BEFORE AND AFTER THE INTRODUCTION OF PENICILLIN*

| | ARRESTED | FAILURES | TOTAL |
|---|---|---|---|
| Penicillin treated | 18 | 10 | 28 |
| Treated before penicillin | 0 | 24 | 24 |
| Total | 18 | 34 | 52 |

*The high statistical significance of these figures has been determined by calculation of chi square from a fourfold table utilizing the correction of Yates for small numbers.[48]

survival from the disease must depend as much on the amount of destruction or deformity of the valve leaflets as on the susceptibility to antibacterial action of the organism in the vegetation.

## SUMMARY

Twenty-eight patients with bacterial endocarditis due to streptococci or staphylococci were treated with penicillin. The patients were unselected except as to the validity of the diagnosis. The beneficial effect of penicillin in these patients is shown by the following considerations:

1. Clinical arrest has occurred in a significant number.
2. The time elapsing since the completion of therapy indicates that the clinical improvement is not temporary.
3. Histopathologic examination of the valve of one patient dying of heart failure after the completion of treatment revealed no bacteria.

Experience in the treatment of these patients re-emphasized the importance of prompt, intensive, and prolonged chemotherapy.

Relapse occurred in two patients after completion of a first course, but both patients responded to a prolonged second course.

Failure in two patients was explained by penicillin-resistant streptococci.

## APPENDIX

CASE 3.—This patient, a 26-year-old white married housewife,* entered the Barnes Hospital for the first time April 17, 1944. At the age of 7 years she had acute arthritis which affected the joints of the arms and legs. The patient had been in bed at that time for three weeks but had subsequently been in excellent health. In July, 1943, she had an induced abortion followed by rather profuse vaginal discharge. On several occasions dilatation and curettage had been carried out. In February, 1944, the patient was given a sulfonamide drug by a local physician apparently because of the vaginal discharge. She took the drug for several weeks but developed oliguria and was sent to a hospital where she remained for five weeks. There she had temperature elevations as high as 102° F., and two weeks before entry here noted that her fingers were becoming clubbed. She had lost approximately thirty pounds during her illness. Two blood cultures taken in the other hospital were reported positive for *Streptococcus viridans.*

On entry, the temperature was 37.8° C.; pulse, 120; respiration, 20; blood pressure, 100/20/0. The patient was a well-developed but poorly nourished white woman who appeared chronically ill. No petechiae were seen. The teeth were carious. The heart was enlarged 11 cm. to the left and 3 cm. to the right. There was a rapid rate with a regular rhythm. At the apex a rumbling diastolic murmur with presystolic accentuation was heard ending in a loud first sound. At the base harsh systolic murmurs and high-pitched early blowing diastolic murmurs were heard; the latter were transmitted along the left sternal border. The splenic

*Reported through the courtesy of Dr. Edward Cannady.

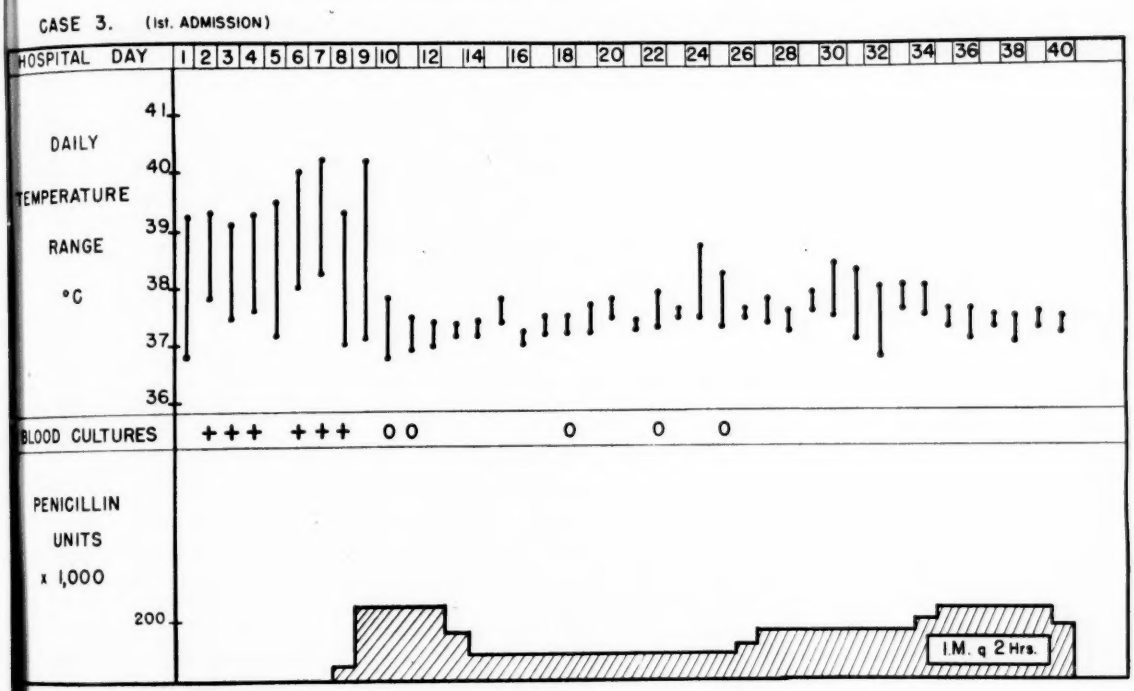
CASE 3. (Ist. ADMISSION)
HOSPITAL DAY
1 2 3 4 5 6 7 8 9 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
DAILY TEMPERATURE RANGE °C
41
40
39
38
37
36
BLOOD CULTURES
+ + + + + + 0 0 0 0 0
PENICILLIN UNITS x 1,000
200
I.M. q 2 Hrs.

Fig. 1.

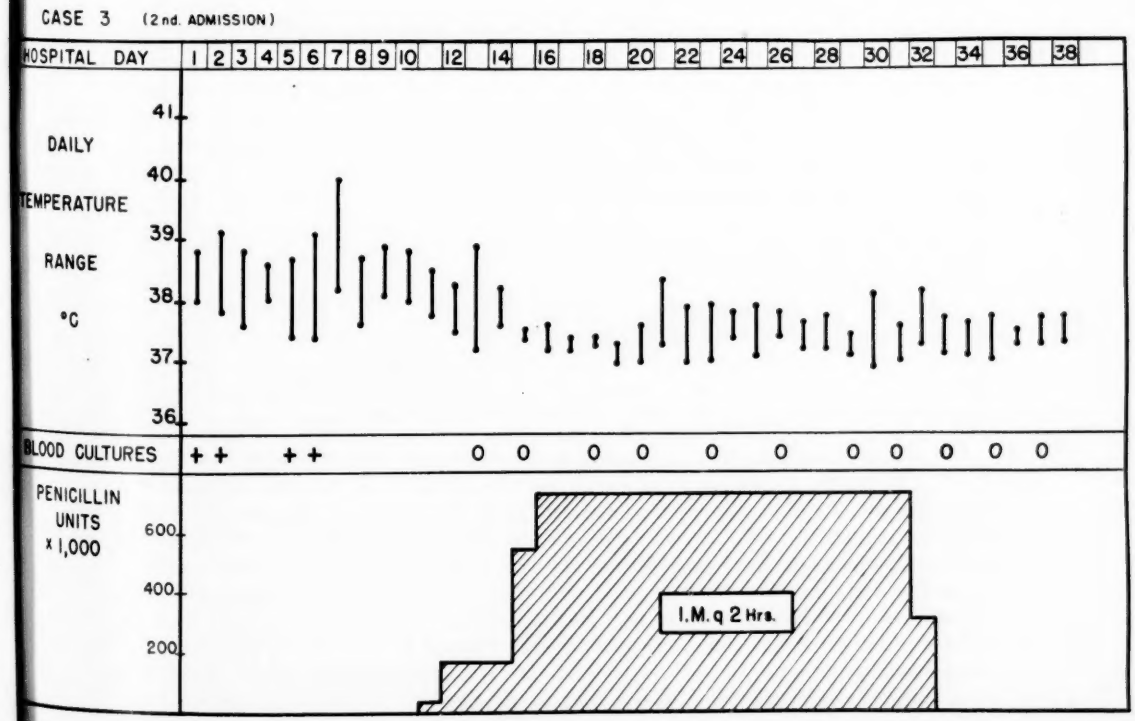
CASE 3 (2nd. ADMISSION)
HOSPITAL DAY
1 2 3 4 5 6 7 8 9 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38
DAILY TEMPERATURE RANGE °C
41
40
39
38
37
36
BLOOD CULTURES
+ + + + 0 0 0 0 0 0 0 0 0 0 0
PENICILLIN UNITS x 1,000
600
400
200
I.M. q 2 Hrs.

Fig. 2.

tip was just palpable. There was clubbing of the fingers and toes and a suggestive splinter hemorrhage was noted under the nail of the left fifth finger. Pelvic examination was not remarkable except for a creamy vaginal discharge. The remainder of the physical examination was concontributory.

The laboratory data revealed that the red blood cell count was 3,600,000 per cubic millimeter with 10 Gm. of hemoglobin per 100 c.c.; the white blood cell count was 8,800, with a normal differential. The urine showed a small amount of albumin but no red cells. Kahn test of the blood was negative. Culture of the vaginal discharge revealed no gonococci. Four consecutive blood cultures were positive for *Streptococcus viridans* with the maximum colony count 320 colonies per cubic centimeter.

Following the report of the positive blood cultures, the administration of 20,000 units of penicillin intramuscularly every two hours was begun (Fig. 1). The temperature, which had been quite septic, fell to within normal limits and remained there throughout the remainder of the hospital course except for occasional elevations above 38° C. The penicillin dosage was lowered to 10,000 units every two hours intramuscularly. Subsequent blood cultures were negative. During the course of penicillin therapy, the patient had repeated episodes of embolic phenomena. The drug was discontinued after thirty days of therapy, at which time the patient had received a total of 4,800,000 units. She left the hospital May 26, 1944, feeling quite well; she was advised to follow her daily temperatures and to report back to the hospital if elevations occurred. On the fourth day after discharge her temperature was noted to be 102° F. and she returned to the hospital for re-evaluation.

*Second Admission.*—Physical examination revealed the temperature to be 38.2° C.; pulse, 70; respirations, 20; blood pressure, 112/44. The examination was essentially as on the previous admission; no petechiae were noted, but the splenic tip was again palpable.

The laboratory findings revealed a persistent anemia and a normal white blood cell count. The urinary sediment showed from 2 to 3 red cells per high-powered field. Three consecutive blood cultures were again positive for *Streptococcus viridans.* Penicillin therapy was instituted with dosages of 20,000 units intramuscularly every two hours (Fig. 2).

The temperature which had ranged between 38° C. and 39° C. for the most part fell to within normal limits within twenty-four hours of the reinstitution of penicillin therapy. The dosage was increased to 60,000 units every two hours intramuscularly and continued at that level for twenty-two days when it was discontinued. At that time the total penicillin given was 12,780,000 units. Subsequent blood cultures were negative; the patient's course was uneventful, and she was discharged afebrile and asymptomatic July 8, 1944. On April 3, 1945, she re-entered for the third time to receive prophylactic penicillin therapy for dental extraction. Her general condition was good, blood cultures were negative, and her hospital course was uneventful. The patient has remained very well.

*Summary.*—A 26-year-old woman who had rheumatic fever at the age of 7 years entered the hospital with subacute bacterial endocarditis due to *Streptococcus viridans.* She received penicillin for thirty days and made a good clinical response, only to relapse four days after discharge. She was then given a second, more intensive course of penicillin, responded well, and has remained in good health since discharge nineteen months ago.

CASE 4.—This patient, a 56-year-old single white man, entered the Barnes Hospital for the first time July 3, 1944, complaining of pain in the left heel and calf for one month and a pruritic rash for three days. At the age of 21 years he had had migratory joint pains and the following year a similar attack. Six months before entry the patient had had oral manipulations apparently designed to make his dentures fit better. One month before entry he developed pain in the left heel which radiated up the calf of the leg and which was accompanied by swelling of the ankles and knees. He then noted palpitation, dyspnea on exertion, and increasing fatigue and consulted a local physician who told him he had "arthritis" and treated him with "arthritis shots." Four days before entry the patient was given sulfathiazole by his physician and on the day before entry he developed a red, itching, maculopapular rash which was generalized.

Physical examination showed a temperature of 38° C.; pulse, 140; respirations, 36; blood pressure, 110/60. The patient was well developed and well nourished and appeared acutely ill. He was slightly cyanotic and markedly short of breath. There was a generalized maculopapular erythematous rash involving the extremities and the trunk, but no petechiae were seen. The neck veins were distended. Bilateral moist basal râles were heard at the lung bases. There were dullness, decreased tactile fremitus, and diminished breath sounds at the right base. The heart was slightly enlarged to the left with a rapid, regular rhythm and sounds of poor quality. Ocular and carotid sinsus pressure did not affect the heart rate. Subsequently, when the rate was slowed, a harsh systolic murmur could be heard at the apex where there was also a low-pitched diastolic rumble; a high-pitched early diastolic murmur was heard along the left sternal border. Examination of the abdomen revealed the liver edge 6 cm. below the costal margin. No other organs or masses were palpated. The remainder of the physical examination was noncontributory.

The laboratory studies showed that the red blood cell count was 4,100,000 with 12 Gm. of hemoglobin; the white blood cell count was 8,900 with a normal differential. Examination of the urine showed no albumin or red cells. The blood Kahn reaction was negative. The blood sulfathiazole level was 0.3 mg. per cent. The venous pressure was 150 mm. of saline; the decholin circulation time, 21 seconds. An electrocardiogram on entry showed auricular flutter with two-one block.

**CASE 4.**

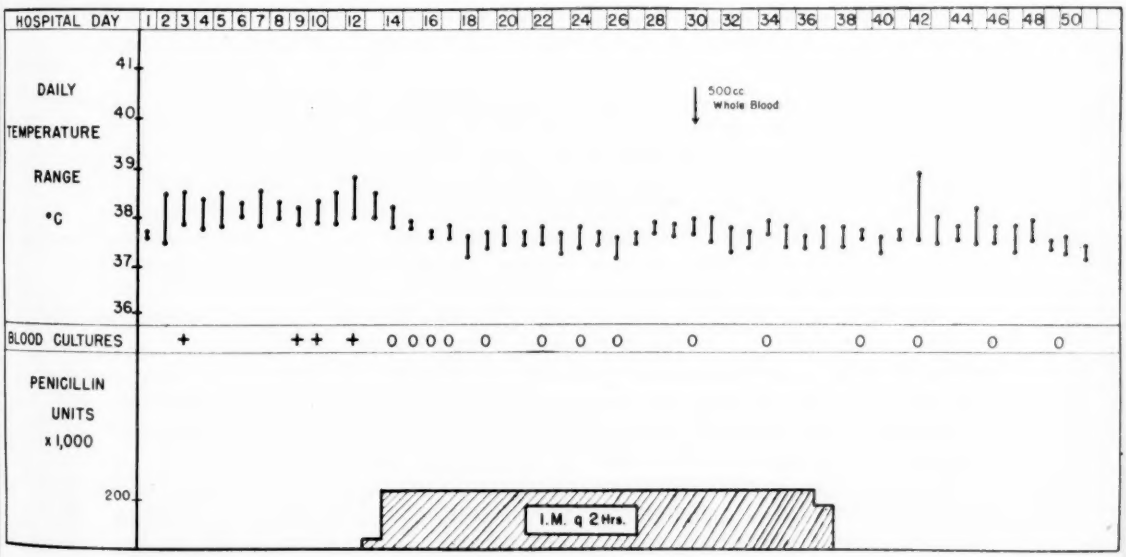


Fig. 3.

The patient was digitalized, and after having transitory auricular fibrillation, the heart reverted to a normal rhythm. Because of continuing low-grade fever, blood cultures were drawn and the first four were positive for *Streptococcus viridans*. The diagnosis of subacute bacterial endocarditis was thus confirmed and the administration of 20,000 units of penicillin intramuscularly every two hours was begun (Fig. 3). There was a good clinical response and all subsequent blood cultures were negative. After twenty-five days of therapy, during which the patient received 5,980,000 units of penicillin, the drug was discontinued. Four days later, the patient complained of pain in the hand, and, although no lesions developed, it was thought that perhaps he had had an embolism. He subsequently was asymptomatic except for marked limitation of his cardiac reserve and he was discharged from the hospital Aug. 23, 1944. He returned to the hospital in one week because of cardiac decompensation but had no symptoms

to suggest recurrence of bacterial endocarditis; repeated blood cultures were negative. He failed to respond to cardiac therapy, however, and gradually failed. He expired Sept. 20, 1944, in an attack of acute pulmonary edema.

At post-mortem examination the heart was found to be very large; the aortic valve was grossly deformed and showed large fungating lesions attached to the valve leaflets. Part of this mass was macerated aseptically and cultured, but no organisms were recovered. The Department of Pathology also cultured the valve, and on the second subculture a growth of *Streptococcus viridans* was obtained. Microscopic examination of the valve, however, showed healing, and bacterial stains of sections of the vegetation and valve showed no organisms.

*Summary.*—A 56-year-old man who had two bouts of rheumatic fever was admitted to the hospital with subacute bacterial endocarditis due to *Streptococcus viridans*. He received a course of penicillin and did well except for a limited cardiac reserve. One month later he died of intractable heart failure without clinical, laboratory, or pathologic evidence of recurrence of the endocarditis.

CASE 6.—This patient, a 22-year-old single white man, was admitted to the Barnes Hospital for the first time Sept. 25, 1944, complaining of weakness and fatigue for seven weeks and afternoon fever for five weeks. At the age of six he had had scarlet fever which left him with a "bad heart." He had been frail and thin ever since, but had apparently gotten along fairly well until two months before entry when he began to notice increasing fatigue and pains in the arms and chest. Five weeks before entry he had a tooth extracted and one week later developed red spots on the finger tips. For about one month he had been short of breath and for two weeks he had had ankle edema. For four days before entry he was given a sulfonamide drug.

Physical examination showed a temperature of 37.8° C.; pulse, 94; respirations, 18; blood pressure, 114/58. The patient was a well-developed, extremely thin young male who looked chronically ill. Petechiae were noted on the palms, finger tips, and dorsum of the feet. The neck veins were not distended. The lungs were clear. The heart was markedly enlarged both to the right and to the left. The rhythm was regular. A loud harsh systolic murmur was heard at the apex where there was also a low-pitched diastolic rumble. The splenic tip could be felt from 4 to 6 cm. below the costal margin. No other abdominal masses were palpable and the remainder of the examination was noncontributory.

The laboratory findings showed that the red blood cell count was 4,320,000; hemoglobin, 12.5 Gm.; white cell count, 9,500; the differential count revealed a shift to the left. The urine showed a small amount of albumin and an occasional red cell. The blood Kahn was positive. The first three blood cultures were negative and the fourth was positive for alpha hemolytic streptococci. Four subsequent blood cultures were likewise positive for alpha hemolytic streptococci.

On the basis of these results the administration of penicillin, 40,000 units intramuscularly every two hours, was started (Fig. 4). The temperature, which had been septic in type until the institution of penicillin therapy, fell to within normal limits thereafter. Penicillin was continued for nineteen days, during which time the patient received 5,850,000 units. Several days before it was discontinued one petechia was noted on the left hand. Subsequent blood cultures were all negative. The patient, as noted, had a positive Kahn test but denied exposure to syphilis; the quantitative Kahn was 40 units. It was felt that nothing more would be done at that time in regard to the question of possible syphilis. The patient left the hospital Nov. 11, 1944.

He did very well and subsequently returned one year later for re-evaluation. At that time physical examination was as before, except that there were no petechiae and the patient had developed an early high-pitched diastolic murmur, heard at the aortic area and along the left sternal border. Repeated blood cultures were negative on this admission, as were the Kahn test and a lumbar puncture.

*Summary.*—A 22-year-old man who had scarlet fever apparently followed by acute rheumatic fever at the age of 6 years entered the hospital because of subacute bacterial endo-

carditis due to *Streptococcus viridans*. He was treated with intramuscular injections of 40,000 units of penicillin every two hours for nineteen days and made a good recovery. He has remained well for fourteen months and is an example of the successful treatment of streptococcal endocarditis with penicillin. The development of aortic incompetency suggests that the aortic valve was the seat of vegetation and, in healing, had become more deformed.

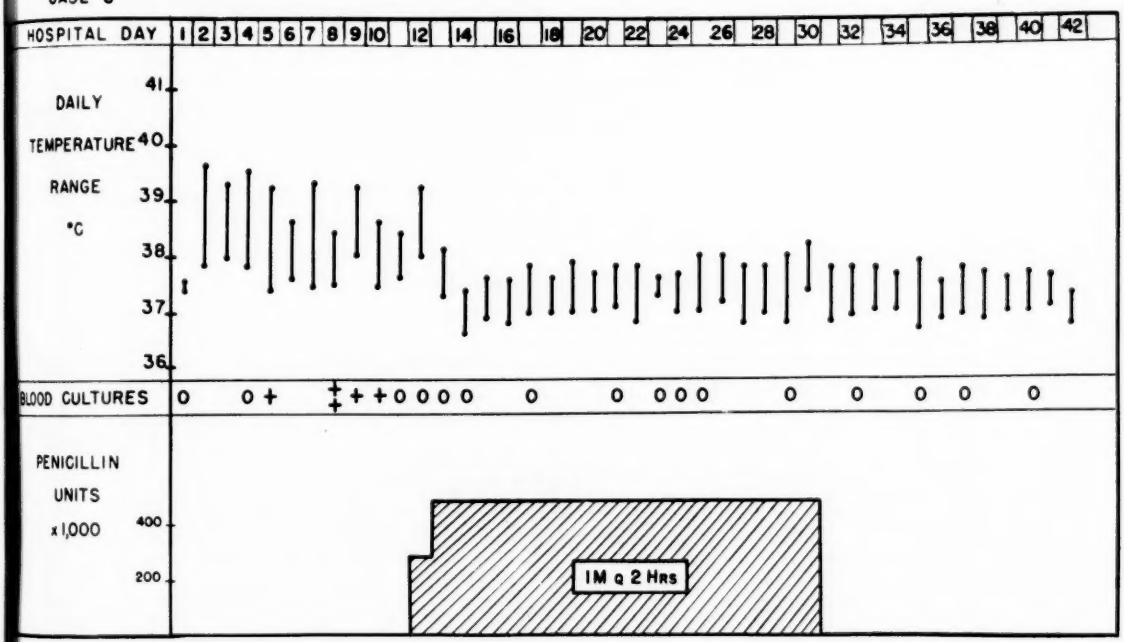


Fig. 4.

CASE 10.*—This patient, a 24-year-old white married housewife, entered the Barnes Hospital for the first time March 12, 1945, complaining of fever, joint pains, and an erythematous rash. The patient had always enjoyed good health and had never had acute rheumatic fever or chorea. About Dec. 1, 1944, three months prior to entry, she had a miscarriage. The fetus was said to have been approximately 6 weeks old, and subsequent to its passage the patient had vaginal bleeding in decreasing amounts for one month; she felt well otherwise until Jan. 1, 1945, when she noted the onset of malaise, weakness, and afternoon fever ranging from 100 to 101° F. She was given a sulfonamide drug, but her symptoms did not improve, and dilatation and curettage were carried out. Subsequent to this procedure the patient was discharged from the hospital in which the operation had been performed, but nine days later she re-entered there and it was found that she had a heart murmur which had not been previously noted. The sulfonamide therapy was continued and the patient was given 10,000 units of penicillin intramuscularly every two hours for nine days. On receiving penicillin, her symptoms cleared, but after the drug was discontinued the temperature rose again and penicillin was reinstituted. Shortly before admission to the Barnes Hospital, the dose was increased to 20,000 units every two hours. During the period that the patient had not been receiving penicillin she had noticed petechiae in the skin and several small splinter hemorrhages under the fingernails. Likewise, when off penicillin she had had some generalized aches and pains which had abated during therapy. The patient had had numerous night sweats, but during the period she had received penicillin, these had decreased. During her illness she had lost about ten pounds.

*Reported through the courtesy of Dr. C. N. Duden.

Physical examination on entry showed a temperature of 37° C.; pulse, 100; respirations, 20; and blood pressure, 110/70. The patient was a well-developed but undernourished female lying quietly in bed in no apparent discomfort. The skin was pale and several petechiae were seen on the third finger of the right hand. The teeth and gums appeared normal except for several petechiae opposite the second right lower molar. The lungs were clear. The heart was enlarged, with the left border of dullness in the fifth interspace, 10.5 cm. from the midsternal line. There was no thrill, but a high-pitched grade 2 systolic murmur could be heard at the apex and was transmitted over the entire precordium. The first sound at the mitral area and the second pulmonic sound were not accentuated. The spleen was not palpable. Pelvic examination was negative. Examination of the extremities revealed marked clubbing of both fingers and toes.

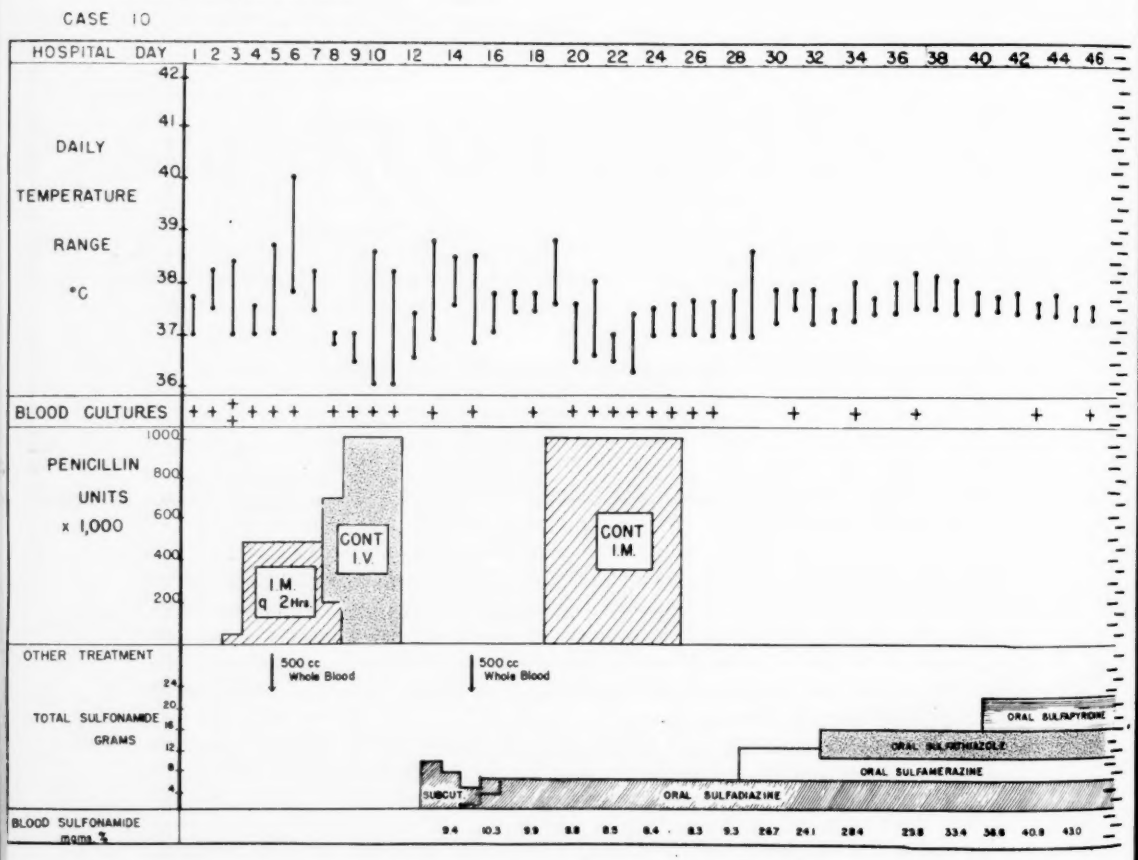


Fig. 5.

The laboratory data showed that the red blood cell count was 4,100,000 with 8 Gm. of hemoglobin. The white blood cell count was 5,600, and the differential count showed a slight left shift. Examination of the urine revealed a small amount of albumin and many red cells. The stool gave a faintly positive test for occult blood. The blood Kahn reaction was negative. The sedimentation rate was 0.2 mm. per minute. Four blood cultures, drawn within the first three hospital days, were all positive for *Streptococcus viridans*, with colony counts ranging from 26 to 170 colonies per cubic centimeter. An electrocardiogram showed only sinus tachycardia. Blood counts were repeated at very frequent intervals and showed a persistent anemia, a normal to slightly elevated white cell count, and often a slight left shift in

the differential count. The urine was free of albumin and rarely showed more than a few red cells. Many urinary sediments showed no red cells at all.

On March 14, 1945, the third hospital day, administration of penicillin, 40,000 units intramuscularly every two hours, was started (Fig. 5). This dose was continued for a little over four days, during which time blood cultures were consistently positive for *Streptococcus viridans* with colony counts as high as 50 or 60 organisms per cubic centimeter. During this period the patient continued to have emboli and her temperature, except for one elevation following transfusion, was of a low-grade variety. On March 19, intravenous penicillin, 1,000,000 units per twenty-four hours, was begun. Despite this increase in dosage, the blood cultures continued to be positive and there were repeated emboli. The route of administration was changed to a continuous intramuscular drip, and although the colony count decreased somewhat, blood cultures were consistently positive. On April 5, 1945, penicillin was stopped. In vitro tests showed this streptococcus to be relatively resistant to penicillin; inhibition of growth was never obtained with less than 3.5 units per cubic centimeter.*

Sulfadiazine, which had been started March 24, was continued, and April 9 sulfamerazine was added in full dosage, blood sulfonamide levels of the order of 25 mg. per cent being obtained. On this regimen the patient's condition remained approximately the same. Blood cultures continued to be positive. On April 13, sulfathiazole in full dosage was added to the list of medications and the total sulfonamide blood level rose to about 30 mg. per cent. On April 22, sulfapyridine likewise was given, so that the patient was receiving four sulfonamides; the combined blood level reached 40 mg. per cent. The patient continued to have a low-grade fever, positive blood cultures, and occasional emboli. At least once there was evidence of cerebral embolization. The sulfonamide blood level got as high as 50 mg. per cent without evidence of renal damage other than that present before any of the drugs were given.[59, 60] Sulfonamide medication was discontinued, however, May 10 because there was no indication of any beneficial effect.

On July 26, penicillin therapy was reinstituted in massive doses by the constant intravenous route, the patient receiving 4,000,000 units every twenty-four hours (Fig. 6). Previous work[2] had suggested that such amounts would be necessary in order to reach blood levels capable of affecting an organism resistant to the degree of this one. Prior to the reinstitution of penicillin, colony counts ranged from 50 to 100 per cubic centimeter of blood. With penicillin therapy, they rapidly fell to an average of about 1 colony per cubic centimeter of blood. Finally, beginning Aug. 1, 1945, the sixth day after the massive doses had been started, blood cultures were entirely negative on three occasions. They then became positive again, although usually only one of the broths, or perhaps one pour plate, would show a colony. Great difficulty was experienced in maintaining the continuous intravenous infusion so the route was changed to hourly intramuscular injections of from 150,000 to 200,000 units. However, this frequent intermittent therapy had to be stopped because of pain, low-grade fever, and signs of inflammation at the sites of injection. An attempt at continuous intrasternal infusion† was unsuccessful because flow could not be maintained. Therefore, the continuous intravenous method was reinstituted and 4,000,000 units of penicillin and 50 mg. of heparin were given in 2,000 c.c. of saline every twenty-four hours.

On the fifth day of this regimen, the patient developed a high septic fever with ranges of temperature from 37.2 to 42° C. During this period the urinary findings were unchanged; blood cultures showed no increase in *Streptococcus viridans*, although three, in addition, showed *Chromobacterium prodigiosum*. The patient's condition rapidly became critical. She was given plasma, sulfadiazine, and sulfamerazine, was digitalized with intravenous lanatoside C; heparin was discontinued. Little change was noted until penicillin was stopped; within six hours the high fever subsided and the patient improved remarkably. It was thought at the time that this stormy period had been a manifestation of sensitivity to penicillin and in

*The organism was also found to have a high in vitro resistance to sulfonamide drugs and to streptomycin.

†Done by Dr. Carl V. Moore.

an attempt to confirm this, intravenous penicillin was again begun Oct. 8, 1945 (Fig. 6). The patient was given 5,000,000 units for two and one-half days, but during this period she developed no fever nor any other signs of toxicity. The drug was not continued because further veins could not be reached, even with surgical intervention. Continuous intramuscular injection could not be done because of local reactions, and it was not feasible to give enough orally to effect the organism.

During the last two months in the hospital, the patient appeared to improve despite the fact that she still had positive blood cultures. She was finally discharged, ambulatory, Oct. 22, 1945. During her hospital stay she had received seven blood transfusions, a high calorie diet, and added vitamins.

Three months after discharge, the patient had gained weight, was maintaining essentially normal activity, and was feeling well.

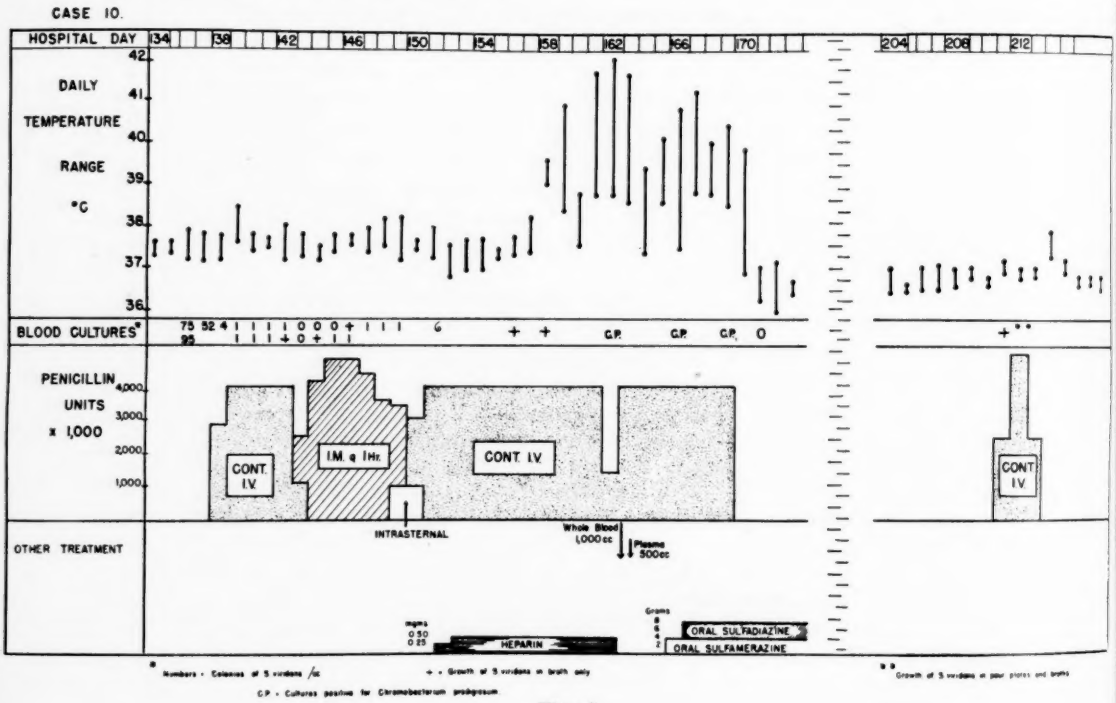


Fig. 6.

*Summary.*—A 24-year-old woman without antecedent history of rheumatic fever or heart disease was admitted to the hospital with subacute bacterial endocarditis due to *Streptococcus viridans.* Following lack of response to usual doses of penicillin and to simultaneous therapy with four sulfonamides, she was given massive doses of penicillin. Although the bacteremia became less intense, it was not controlled, and the patient was discharged improved but with blood cultures still positive.

CASE 21.—This patient, a 22-year-old white married woman, entered the Barnes Hospital Aug. 30, 1945, complaining of fever, anemia, weight loss, and pain in the back and legs. The patient gave no past history of definite acute rheumatic fever, although in her youth she had had episodes of pain in her legs. Until the onset of the present illness she had been entirely unaware of any cardiac disease. She had been apparently well until the summer of 1944, at which time she had had a tooth extracted; an abscess developed at the site of extraction and the patient felt poorly for several months, although she continued to be up

and about and took care of her household duties. Ten months before entry she developed a swelling in the tip of her left index finger, and a similar lesion appeared in the right large toe. Both sites were very painful to touch but subsided in several weeks. In February, 1945, the patient had a "nervous breakdown," during which time she was apparently depressed. In

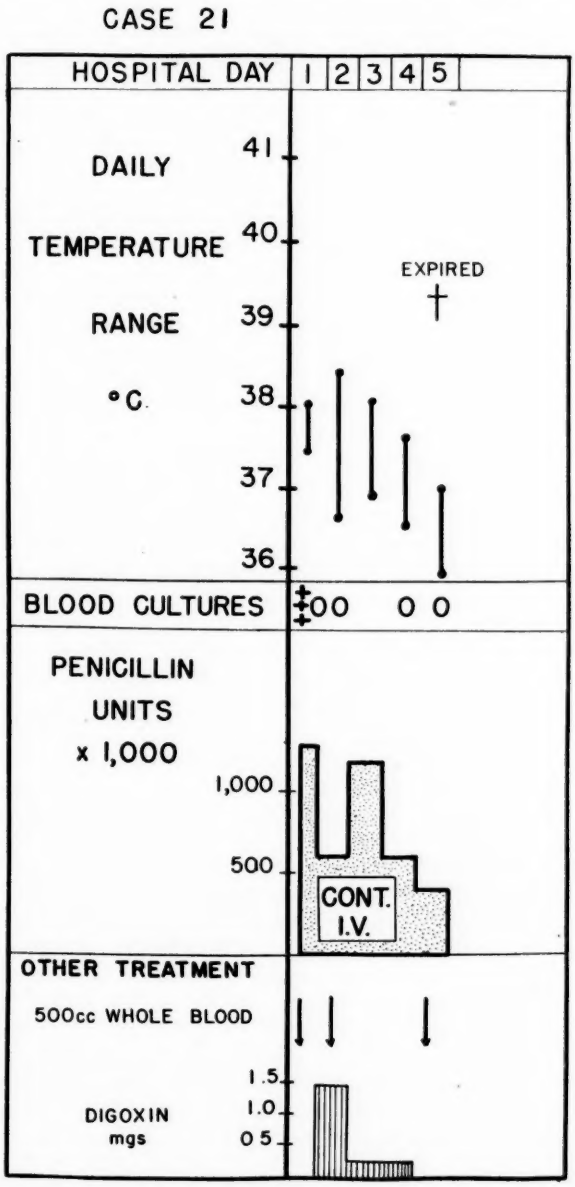


Fig. 7.

March, 1945, she was told by her local physician that she was anemic; she felt weak and became progressively more tired. Because of the development of severe pain in both flanks, she finally went to a hospital where she was told she had an infected kidney and a leaky heart. She was given penicillin for three days and then discharged. From that time on she became gradually

worse, and for a month before entry had not seen any doctor because of discouragement with previous treatment. For several days before entry here she had severe pain in both legs, worse in the right.

Physical examination on admission showed a temperature of 38° C.; pulse, 120; respiration, 28; blood pressure, 92/60. The patient appeared in extremis. The skin was dry, there was marked wasting, and great numbers of petechiae were seen over the extremities in the trunk. Many petechiae were observed in the conjunctivae and the sclerae, and numerous fresh hemorrhages were seen in the optic fundi. The mucous membranes of the mouth likewise showed petechiae. The neck was not stiff. The posterior cervical lymph nodes were enlarged. Examination of the chest revealed medium and fine inspiratory râles over the left lower lobe, but the remainder of the chest was clear and there was no friction rub. The heart was enlarged with a diffuse apical impulse in the sixth interspace outside the mid-clavicular line. A presystolic thrill was felt at the apex. The sounds were rapid and of poor quality except that the second pulmonic sound was accentuated. A harsh, loud systolic murmur could be heard at the apex and was transmitted to the axilla and over the entire precordium. There was also a rumbling mid-diastolic murmur at the apex. The abdomen was tense, but the liver edge could be felt 6 cm. below the costal margin and was slightly tender. The spleen was not palpable. There was bilateral costovertebral angle tenderness. The fingers were clubbed. Bilateral ankle edema was noted. The right foot was cold and the toes were blue. Both femoral arteries pulsated well, but no popliteal or dorsalis pulsations could be felt on the right.

The laboratory data showed that the red blood cell count was 2,800,000 with 7.5 Gm. of hemoglobin. The white cell count was 31,000, with a moderate left shift in the differential. The urine showed a moderate amount of albumin and the sediment contained from one to two red cell casts, 30 to 40 white cells, and 10 red cells per high-power field. The Kahn test was negative. Three blood cultures taken at ten-minute intervals shortly after admission were all positive for *Streptococcus viridans*. The nonprotein nitrogen was 36 mg. per cent; the total proteins, 4.8 Gm., with 2.5 Gm. of albumin and 2.3 Gm. of globulin. An electrocardiogram showed myocardial damage and sinus tachycardia. Following digitalization with intravenous digoxin there was partial heart block with prolonged A-V conduction, frequent blocked ventricular beats, and excess digitalis effect. Two sedimentation rates were within normal limits.

Immediately after the blood cultures were obtained, intravenous penicillin was begun, and during the first hour the patient received 150,000 units (Fig. 7). A continuous intravenous drip was maintained, and she received approximately 1,000,000 units of the drug daily. Within eleven hours after the institution of penicillin therapy the blood culture was negative, and during the remaining four days of life four other blood cultures were negative. The white count remained elevated. The venous pressure stayed within normal limits until the third of three blood transfusions was being given, at which time it rose and the transfusion had to be discontinued. Despite the intensive therapy the patient developed new petechiae and a left pleural friction rub was noted. She became stuporous and then comatose and died on the morning of Sept. 3, 1945. Her temperature never rose above 38.4° C. and terminally fell to a subnormal level. At the time of death she had received 4,150,000 units of penicillin.

*Summary.*—A 22-year-old woman entered the hospital in extremis with overwhelming subacute bacterial endocarditis due to *Streptococcus viridans* and recent arterial embolization. Despite penicillin therapy which sterilized the blood stream within eleven hours, the patient died on the fourth hospital day.

CASE 25.*—This patient, a 17-year-old white single schoolgirl, was admitted to the Barnes Hospital for the first time March 23, 1944, because of chills and fever of six days' duration. At the age of 11 years she had had acute rheumatic fever and since that time had been known to have a heart murmur. She had had been quite well, however, until one month before entry, when her left ear began to drain. Aside from two transient episodes of pain

*Reported through the courtesy of Dr. H. L. Alexander.

in the ear two weeks before entry, she had had no other symptoms. Six days before admission she had several shaking chills and her temperature rose to 105° F. She complained of pain in the right hip and in the fourth finger of the left hand and felt weak and exhausted. She was admitted to a hospital where two blood cultures were said to show "diplococci resembling pneumococci." She was given sulfadiazine and three blood transfusions but continued to have a high septic fever and to do poorly. She was transferred to the Barnes Hospital for further treatment.

Physical examination showed a temperature of 40.8° C.; pulse, 122; respiration, 26; and blood pressure, 130/64. The patient was a well-developed, well-nourished young woman who appeared acutely and critically ill. She complained only of feeling "chilly." Her skin was warm and moist; several petechiae were present on the hands and feet. Bloody crusts were

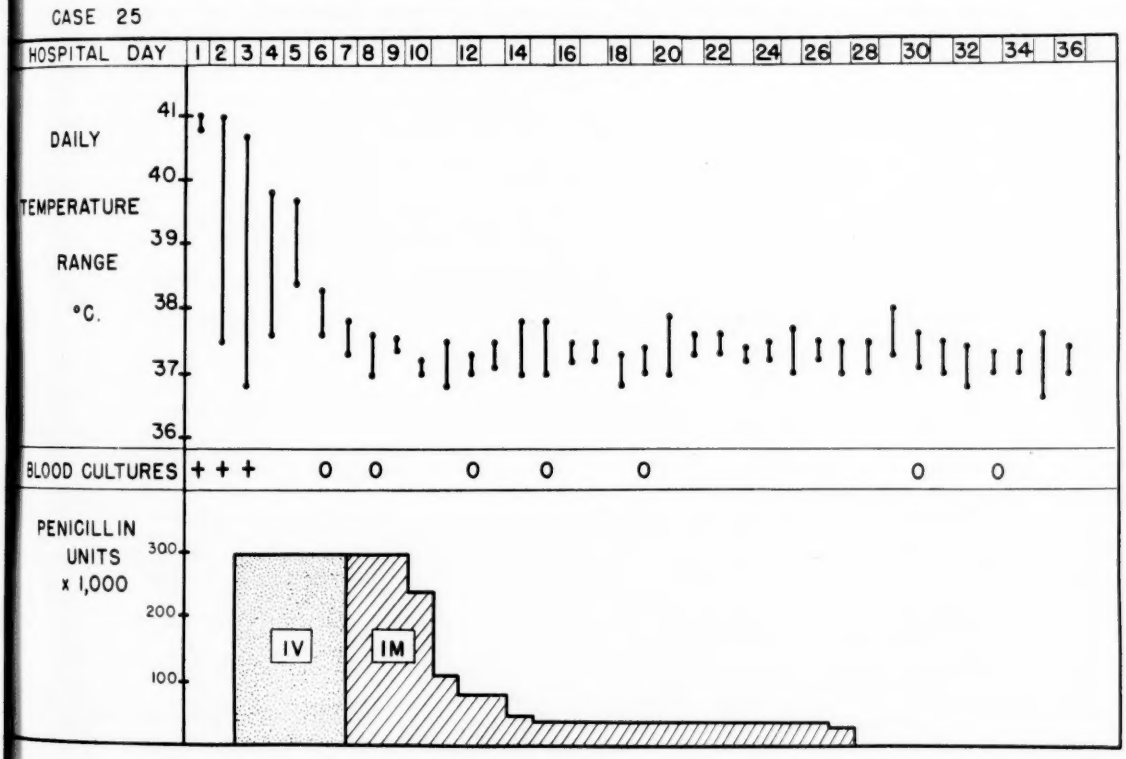


Fig. 8.

noted in both nostrils. The lungs were clear. The heart was slightly enlarged to the left in the fifth interspace; the rate was rapid and the rhythm was regular. The second pulmonic sound was greater than the second aortic sound. A rough high-pitched systolic murmur was heard maximally at the apex, but it was transmitted over all of the precordium. The tip of the spleen was just barely palpable at the costal margin. No other organs or masses were felt in the abdomen. The remainder of the physical examination was not remarkable.

Laboratory data revealed that the red blood cell count was 4,400,000 with 15 Gm. of hemoglobin. The white cell count was 10,500, with a marked left shift. The urine showed no albumin or red cells. The blood Kahn test was negative. Two blood cultures taken on entry were both positive for hemolytic *Staphylococcus aureus*, the maximal colony count being 140 per cubic centimeter. The organism fermented mannite and was coagulase positive.

On the evening of the second hospital day, penicillin was begun in doses of 12,500 units every hour; the drug was injected into the tubing of a continuous intravenous drip (Fig. 8).

On the following day the dose was changed to 25,000 units every two hours and this regimen was continued for five days. The temperature, which had shown marked swings from 37.5 to 41° C., began to respond within forty-eight hours of the institution of penicillin therapy and by the fifth day had fallen to normal limits. At that time administration was changed to the intramuscular route and all subsequent penicillin was given this way. During the first two or three hospital days the patient continued to have embolic phenomena and an occasional red cell was found in the urinary sediment, but otherwise her hospital course was very uneventful. The temperature remained normal and the penicillin dose was gradually decreased so that in the last four days she received only 5,000 units every three hours. All blood cultures taken after institution of penicillin therapy were negative and the patient left the hospital April 28, 1944. She has been exceedingly well in the twenty-one months since discharge.

*Summary.*—A 17-year-old girl with known rheumatic heart disease of six years' duration was admitted to the hospital with acute bacterial endocarditis due to hemolytic *Staphylococcus aureus.* She was treated with relatively small doses of penicillin for twenty-six days and responded dramatically. She has remained well since discharge, twenty-one months ago.

Acknowledgment is made to Miss Brigitte Watty for preparation of the charts.

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# MAINTENANCE OF THERAPEUTIC BLOOD CONCENTRATIONS OF PENICILLIN FOR TWENTY-FOUR HOURS FOLLOWING SINGLE INJECTIONS OF PENICILLIN-BEESWAX-PEANUT OIL MIXTURES

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INTRAMUSCULAR injection of penicillin-beeswax-peanut oil mixtures is one of the most promising methods so far described for prolongation of the action of penicillin in the body.[1] Optimal results are obtained with mixtures containing approximately 5 per cent beeswax and 300,000 units of penicillin per cubic centimeter.[2] Such mixtures are now available commercially.

Ideally, it should be possible to administer an average day's supply of penicillin in a single injection of a penicillin-beeswax-peanut oil mixture, with maintenance of effective therapeutic concentrations in the blood stream for a period of twenty-four hours. Unfortunately, this objective has not been attained with single intramuscular injections of 300,000 units (1 c.c.) of penicillin in beeswax-peanut oil.[3] In a series of patients studied in this hospital, assayable levels were present for from eight to twenty-eight hours, but in approximately two-thirds of the individuals penicillin could be detected for no longer than twelve hours.[3] The results were considerably better when the material was injected subcutaneously rather than intramuscularly, assayable levels then being present for twenty hours in two-thirds of twenty-five patients.[3]

The purpose of the present report is to describe the results of assays of the blood performed every four hours for thirty-six hours following single intramuscular and subcutaneous injections of 600,000 units (2 c.c.) of a commercially prepared penicillin-beeswax-peanut oil mixture. After warming the mixture in the incubator at 37° C. for from sixty to ninety minutes, 2.2 c.c. were aspirated into a 5 c.c. Luer-Lok syringe through a No. 15 needle; a No. 17 needle was used for the injection into the tissues. Intramuscular injections were made in the upper outer quadrant of the buttock. Subcutaneous injections were made either over the insertion of the deltoid muscle, or along the lateral aspect of the thigh, overlying the fascia lata. The individuals used for the study were all healthy young men suffering from acute gonococcic urethritis; twenty patients received the mixture intramuscularly and twenty subcutaneously (eleven over the deltoid muscle and nine along the lateral aspect of the thigh). As in the earlier studies, moderate soreness was noted at the site of the injection for from twenty-four to forty-eight hours and was less severe following subcutaneous than following intramuscular injections. No other local or systemic manifestations of toxicity were observed, and it may especially be noted that with the subcutaneous route no induration, nodule formation, or sterile abscesses occurred. It should be

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emphasized, however, that the mixture must be injected well down into the subcutaneous tissue, since absorption is impaired and nodules may develop if the material is inserted into the skin itself. Penicillin assays were performed by the method of Kirby and Rantz.[4]

Results of the assays are presented in Tables I and II. Only the levels obtained frow twelve to thirty-six hours after the injections are included; in about one-half of the patients assays were also made at one-half, one, four, and

TABLE I. BLOOD LEVELS OF PENICILLIN (UNITS PER CUBIC CENTIMETER) IN TWENTY PATIENTS FOLLOWING SINGLE INTRAMUSCULAR INJECTIONS OF 600,000 UNITS (2 C.C.) OF A PENICILLIN-BEESWAX-PEANUT OIL MIXTURE

| CASE | HOURS | | | | | | | TOTAL DURATION |
|---|---|---|---|---|---|---|---|---|
| | 12 | 16 | 20 | 24 | 28 | 32 | 36 | |
| 1 | 0.2 | 0.1 | 0 | 0 | 0 | 0 | 0 | 16 |
| 2 | 0 | .05 | 0 | 0 | 0 | 0 | 0 | 16 |
| 3 | 0.1 | 0.1 | 0.1 | 0 | 0 | 0 | 0 | 20 |
| 4 | 0.2 | 0.2 | 0.1 | 0 | 0 | 0 | 0 | 20 |
| 5 | .1 | .2 | .1 | 0 | 0 | 0 | 0 | 20 |
| 6 | .2 | .4 | .1 | 0 | 0 | 0 | 0 | 20 |
| 7 | .4 | .1 | .1 | 0 | 0 | 0 | 0 | 20 |
| 8 | .1 | .1 | .07 | 0 | 0 | 0 | 0 | 20 |
| 9 | 1.0 | .2 | .2 | 0 | 0 | 0 | 0 | 20 |
| 10 | .2 | .2 | .2 | .1 | 0 | 0 | 0 | 24 |
| 11 | 0.1 | .07 | .2 | .1 | 0 | 0 | 0 | 24 |
| 12 | .07 | .2 | .07 | .2 | 0 | 0 | 0 | 24 |
| 13 | .2 | .07 | .07 | .05 | 0 | 0 | 0 | 24 |
| 14 | 0.1 | .2 | .05 | .2 | 0 | 0 | 0 | 24 |
| 15 | .1 | .2 | .7 | .07 | 0 | 0 | 0 | 24 |
| 16 | .2 | .1 | .1 | .07 | 0 | 0 | 0 | 24 |
| 17 | .2 | .2 | .2 | .2 | 0 | 0 | 0 | 24 |
| 18 | .4 | .2 | .1 | .2 | .07 | 0 | 0 | 28 |
| 19 | .4 | .2 | .1 | .1 | 0 | .04 | 0 | 32 |
| 20 | .2 | .2 | .2 | .2 | .1 | .2 | .1 | 36 |

TABLE II. BLOOD LEVELS OF PENICILLIN (UNITS PER CUBIC CENTIMETER) IN TWENTY PATIENTS FOLLOWING SINGLE SUBCUTANEOUS INJECTIONS OF 600,000 UNITS (2 C.C.) OF A PENICILLIN-BEESWAX-PEANUT OIL MIXTURE

| CASE | SITE (ARM OR THIGH) | HOURS | | | | | | | TOTAL DURATION |
|---|---|---|---|---|---|---|---|---|---|
| | | 12 | 16 | 20 | 24 | 28 | 32 | 36 | |
| 1 | A | .5 | .2 | .1 | 0 | 0 | 0 | 0 | 20 |
| 2 | A | .2 | .2 | .1 | 0 | 0 | 0 | 0 | 20 |
| 3 | A | .2 | .1 | .07 | 0 | 0 | 0 | 0 | 20 |
| 4 | A | .2 | .4 | .2 | .05 | 0 | 0 | 0 | 24 |
| 5 | A | .2 | .2 | .1 | .1 | 0 | 0 | 0 | 24 |
| 6 | T | .5 | .2 | .1 | .1 | 0 | 0 | 0 | 24 |
| 7 | T | .2 | .2 | .1 | .1 | 0 | 0 | 0 | 24 |
| 8 | A | .1 | .7 | .1 | .05 | .07 | 0 | 0 | 28 |
| 9 | A | .2 | .2 | .7 | .2 | .1 | 0 | 0 | 28 |
| 10 | A | .2 | .2 | .4 | .2 | .04 | 0 | 0 | 28 |
| 11 | T | .1 | .1 | .07 | .1 | .07 | 0 | 0 | 28 |
| 12 | T | .7 | .2 | .5 | .1 | .1 | 0 | 0 | 28 |
| 13 | A | .1 | .04 | .2 | .05 | 0 | .1 | 0 | 32 |
| 14 | A | .2 | .2 | .2 | .1 | .07 | .04 | 0 | 32 |
| 15 | T | .5 | .1 | .1 | .1 | .2 | .05 | 0 | 32 |
| 16 | T | .7 | .2 | .5 | .2 | .07 | .1 | 0 | 32 |
| 17 | A | .2 | .2 | .07 | 0 | .07 | 0 | .04 | 36 |
| 18 | T | .7 | .2 | .4 | .1 | .1 | .1 | .05 | 36 |
| 19 | T | .7 | .2 | .2 | .2 | .2 | 0 | .04 | 36 |
| 20 | T | .2 | .2 | .5 | .2 | .2 | .2 | .2 | 36 |

eight hours, with results similar to those obtained in the earlier studies with 300,000 units. Measurable concentrations of penicillin were present for from sixteen to thirty-six hours following intramuscular injection and for from twenty to thirty-six hours following subcutaneous administration. The results following subcutaneous administration were considerably better, from the standpoint of prolongation of assayable blood levels, than were those following intramuscular administration. Following intragluteal injections, nine patients had measurable concentrations for no longer than twenty hours, and in two of these the duration was only sixteen hours. With the subcutaneous route, on the other hand, assayable levels were present for less than twenty-four hours on only three occasions, and in none of these was the duration less than twenty hours. In other words, with the subcutaneous route, measurable levels were present for twenty-four hours or longer in 85 per cent of the patients, while with the intramuscular route twenty-four-hour levels were obtained in only a little over one-half of the patients, or 55 per cent. The results with subcutaneous administration appeared equally as good, and possibly a little better, when the material was injected along the lateral aspect of the thigh than when it was administered in the region of the deltoid.

The height of the blood levels was much the same with both the subcutaneous and intramuscular routes. With the subcutaneous, eighty determinations were 0.1 unit per cubic centimeter or higher, and eighteen ranged from 0.04 to 0.1 unit per cubic centimeter, with the intramuscular route, sixty levels were 0.1 u. per cubic centimeter or higher, and thirteen varied from 0.04 to 0.1 unit per cubic centimeter.

It is apparent, then, that in most instances effective therapeutic concentrations of penicillin are present in the blood stream for twenty-four hours or longer following single subcutaneous injections of 600,000 units of penicillin in beeswax-peanut oil. Since absorption is equally good from the subcutaneous tissue of the thigh and the arm, many sites of injection are available in patients requiring prolonged treatment. From the height of the levels, it seems probable that most patients with infections can be treated successfully with a single daily injection. The fact that larger amounts of penicillin are required than with penicillin in saline is of little importance in view of the abundant supplies of penicillin now available and is more than compensated for by the convenience of being able to administer an entire day's treatment with a single subcutaneous injection.

## SUMMARY

Effective therapeutic concentrations of penicillin are maintained in the blood stream for twenty-four hours or longer following single subcutaneous injections of 600,000 units of penicillin in beeswax-peanut oil. Results following intramuscular injections are somewhat less satisfactory than with the subcutaneous route.

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# THE CONCENTRATION OF PENICILLIN IN THE SPINAL FLUID FOLLOWING INTRAMUSCULAR ADMINISTRATION IN NEUROSYPHILIS

## A Negative Report

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THE excretion and persistence of penicillin in the spinal fluid under various conditions has been a question of considerable interest. In the early investigation of the absorption, excretion, and distribution of penicillin, Rammelkamp and Keefer[1] found none in the spinal fluid of two normal adults after intravenous injections of 10,000 units; one tested up to forty-five minutes after the injection, and the other up to 195 minutes. Herrell[2] administered as much as 30,000 units of penicillin intravenously in a period of fifteen minutes to a patient who did not have any demonstrable lesions of the central nervous system and could not detect any penicillin in the spinal fluid removed thirty and sixty minutes after the injection.

Penicillin injected intrathecally is slowly absorbed from the subarachnoid space and can be detected in the spinal fluid thirty-one hours later. More rapid absorption occurs from the spinal fluid of patients with meningitis, but the agent can still be found in significant amounts twenty-four hours after intraspinal administration. Because of the evidence that penicillin did not readily penetrate the barrier from blood to spinal fluid, Rosenberg and Arling,[3] in a large series of patients with meningitis, utilized a combination of intrathecal with intravenous and/or intramuscular penicillin therapy. However, they were among the first to suggest that, when penicillin would ultimately be prepared in a more concentrated and a more highly purified form, free from pyrogens, it might be excreted into the subarachnoid space in sufficient amounts following intramuscular or intravenous administration to justify the abandonment of intrathecal therapy. Dawson and Hobby[4] mentioned that, although in normal persons little or no penicillin is excreted in the cerebrospinal fluid, detectable amounts may be found in the presence of active inflammation. No data supporting their statement was offered at the time.

In August, 1944, Rosenberg and Sylvester[5] first reported detectable concentrations of penicillin in the spinal fluid after intravenous and intramuscular administration in eight patients with meningitis. In these patients, penicillin was administered in doses of from 20,000 to 40,000 Oxford units, and from 60 to 140 minutes later penicillin was found in the spinal fluid in concentrations varying

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from 0.03 to 0.35 unit per cubic centimeter. This report was the first to demonstrate that penicillin administered other than intrathecally, in adequate dosages, may be effective in the treatment of meningitis without necessitating the supplementary route of administration. Confirmatory results were published by Cooke and Goldring,[6] whose observations were conducted on infants from 4 to 34 months of age receiving relatively much larger doses of penicillin (from 500 to 5,000 units per kilogram of body weight) than usually given to adults. Nine patients were included in their study, five of whom had acute meningitis; three, acute nephritis; and one, acute pericarditis. All were given penicillin intramuscularly, except one patient who received an intraperitoneal injection In four of the nine patients, from 0.1 to 0.4 unit of penicillin per cubic centimeter was found in the spinal fluid from fifteen minutes to one hour after the intramuscular injection. All of these patients had acute meningitis. In the one patient given penicillin intraperitoneally, 0.2 unit per cubic centimeter was found four hours after the injection. These observations illustrated that, when relatively large doses of penicillin are given intramuscularly, the drug may be found in the spinal fluid within fifteen minutes to one hour but tends to disappear after one and one-half hours.

In spite of the fact that penicillin has been actively used in the therapy of neurosyphilis during the past few years, there have been no published reports of studies on its excretion in the spinal fluid in patients so treated. If inflammatory changes, such as those existing in cases of meningitis, are responsible for the increased penetration of penicillin into the spinal fluid, it would not seem too unlikely that in neurosyphilis, with its various combinations of meningeal, vascular, and parenchymatous central nervous system changes, similarly increased concentrations of penicillin might be obtained and the necessity for cumbersome intrathecal administration avoided. There is, of course, also the possibility that since neurosyphilis is believed by many to be primarily a vascular disease, the penetration of penicillin through the blood-spinal fluid barrier may not actually be necessary.

In an effort to determine whether penicillin significantly penetrates into the spinal fluid in neurosyphilitic patients with different spinal fluid syndromes, this study was undertaken.

## MATERIAL AND METHOD

The routine course of therapy at this neurosyphilis center from May to November, 1945, consisted of the simultaneous administration of therapeutic malaria and 3,600,000 units of penicillin over a period of fifteen days. Penicillin, in doses of 30,000 units, was injected intramuscularly every three hours, day and night, until a total of 120 injections had been given. This therapy was not begun until the patient developed his first elevation of temperature above 102° F. from the malaria infection. Twelve patients were included in the study, and a total of twenty-five observations was made. Since the degree of involvement of the central nervous system in neurosyphilis conceivably has an effect on the concentration of penicillin in the spinal fluid, cases of both asymptomatic and symptomatic neurosyphilis (meningovascular, tabes dorsalis, and dementia

paralytica) were included. Similarly, since the active meningeal inflammation, as in the previously reported cases of bacterial meningitis, probably greatly influences the distribution of the drug, both active (elevated cell counts) and inactive spinal fluid syndromes were chosen for the investigation. Specimens of blood, spinal fluid, and urine were obtained for penicillin assay at different intervals from 10 to 150 minutes following the intramuscular injection of 30,000 units. Most of the patients had previously received between 1,800,000 and 3,510,000 units before these observations were recorded.

*Method of Penicillin Assay.—*

*Materials Used:* Sterile saline; sterile pipettes (0.1 c.c. and 1 c.c.); chemically clean Wassermann and Loeffler's tubes; stock solution of penicillin (10,000 units per cubic centimeter); Wassermann racks; sterile brain-heart infusion broth (4 c.c. per tube); a known sensitive organism *(Staphylococcus aureus hemolyticus)*—a broth suspension of approximately 600,000 per cubic centimeter was used as the inoculum.

*Determination of the Organism Sensitivity:* Thirteen tubes of sterile brain-heart infusion broth were placed in a rack. Penicillin was added to each tube in the amounts shown in Table I.

TABLE I

| TUBE | PENICILLIN (UNITS) |
|---|---|
| 1 | 0.15 |
| 2 | 0.20 |
| 3 | 0.25 |
| 4 | 0.30 |
| 5 | 0.40 |
| 6 | 0.50 |
| 7 | 0.60 |
| 8 | 0.80 |
| 9 | 1.0 |
| 10 | 1.5 |
| 11 | 2.5 |
| 12 | 5.0 |
| 13 | None Control |

Each tube was inoculated with 0.1 c.c. of a broth suspension of *Staph. aureus* and incubated for twenty-four hours at 37° C. The organism was found to be sensitive to 0.25 unit of penicillin.

*Procedure:* Ten tubes of sterile brain-heart infusion broth were placed in a rack. The body fluid was added to the tubes in the amounts shown in Table II.

TABLE II

| TUBE | BODY FLUID (C.C.) |
|---|---|
| 1 | 0.1 |
| 2 | 0.2 |
| 3 | 0.3 |
| 4 | 0.5 |
| 5 | 0.8 |
| 6 | 1.0 |
| 7 | 1.3 |
| 8 | 1.5 |
| 9 | 2.0 |
| 10 | None Control |

Each tube was inoculated with 0.1 c.c. of broth suspension of *Staph. aureus*. The racks were incubated for twenty-four hours at 37° C. The racks were removed at the end of incubation and read. The tube which contained the smallest amount of body fluid and contained no growth was taken to indicate the amount of penicillin present. (Example: No growth in Tube 4; therefore, 0.5 c.c. of body fluid equals 0.25 unit of penicillin; 1 c.c. equals 0.5 unit.)

TABLE III. PENICILLIN CONCENTRATION IN BODY FLUIDS FOLLOWING INTRAMUSCULAR ADMINISTRATION IN NEUROSYPHILIS

| | | CEREBROSPINAL FLUID | | | NUMBER OF MINUTES AFTER 30,000 UNITS I.M. | | | |
|---|---|---|---|---|---|---|---|---|
| CASE | CLINICAL DIAGNOSIS | CELL COUNT (PER C.MM.) | TOTAL PROTEIN (MG. %) | WASS. | | BLOOD SERUM (CONC.*) | SPINAL FLUID (CONC.) | URINE (CONC.) |
| 1 | Asymptomatic | 51 | 28.0 | +0.25 | 60 | — | -.25 | — |
| 2 | Tabes dorsalis | 0 | 40.0 | +0.5 | 90 | — | -.25 | — |
| 3 | Asymptomatic | 10 | 38.5 | +0.25 | 30 | -.25 | -.25 | — |
| 4 | Asymptomatic | 1 | 23.0 | +0.5 | 150 | -.125 | -.125 | — |
| 5 | Asymptomatic | 3 | 45.0 | +0.5 | 120 | -.25 | -.125 | — |
| 6 | Asymptomatic | 3 | 19.0 | +0.5 | 60 | — | -.125 | — |
| 7 | Meningovascular | 1 | 45.0 | +0.1 | 30 | — | -.125 | — |
| 8 | Dementia paralytica | 46 | 64.0 | +0.1 | 100 | — | -.125 | — |
| 8 | Dementia paralytica | 46 | 64.0 | +0.1 | 20 | +.7 | -.125 | — |
| 8 | Dementia paralytica | 46 | 64.0 | +0.1 | 45 | +1.0 | -.125 | — |
| 8 | Dementia paralytica | 46 | 64.0 | +0.1 | 70 | — | — | +2.5 |
| 9 | Meningovascular | 9 | 45.0 | +0.5 | 60 | — | -.125 | — |
| 9 | Meningovascular | 9 | 45.0 | +0.5 | 10 | +.6 | -.125 | — |
| 9 | Meningovascular | 9 | 45.0 | +0.5 | 40 | +1.3 | -.125 | — |
| 9 | Meningovascular | 9 | 45.0 | +0.5 | 65 | — | — | +2.5 |
| 10 | Asymptomatic | 25 | 92.0 | +0.1 | 90 | — | -.125 | — |
| 10 | Asymptomatic | 25 | 92.0 | +0.1 | 50 | +.7 | -.125 | — |
| 10 | Asymptomatic | 25 | 92.0 | +0.1 | 70 | +1.3 | -.125 | — |
| 10 | Asymptomatic | 25 | 92.0 | +0.1 | 85 | — | — | +2.5 |
| 11 | Asymptomatic | 1 | 23.0 | +0.25 | 40 | +.7 | -.125 | — |
| 11 | Asymptomatic | 1 | 23.0 | +0.25 | 65 | +1.0 | -.125 | — |
| 11 | Asymptomatic | 1 | 23.0 | +0.25 | 80 | — | — | +2.5 |
| 12 | Asymptomatic | 64 | 38.0 | +0.1 | 30 | +.7 | -.125 | — |
| 12 | Asymptomatic | 64 | 38.0 | +0.1 | 55 | +1.0 | -.125 | — |
| 12 | Asymptomatic | 64 | 38.0 | +0.1 | 75 | — | — | +2.5 |

*Conc., Concentration of penicillin in body fluid. For example, +.7 indicates presence of penicillin in .7 unit per cubic centimeter; -.125 indicates no penicillin within the limits of sensitivity of the tests employed (less than .125 unit per cubic centimeter).

This technique would detect a penicillin concentration as low as 0.125 unit per cubic centimeter. In Table III, a plus sign before the recorded concentration indicates the presence of the drug in that amount. A minus sign before the observation indicates that *no* penicillin was detected in the concentration tested. Body fluids from an untreated patient were used as negative controls. Body fluid treated with the stock penicillin was used as positive control. This procedure was applicable to urine, blood, and spinal fluid. The assays were done immediately after the collection of specimens.

## RESULTS AND DISCUSSION

Of the twelve patients included in this investigation, eight had asymptomatic neurosyphilis and showed various degrees of inflammatory spinal fluid activity represented by elevated cell counts from 1 to 64 cells per cubic millimeter.

Two were patients with meningovascular neurosyphilis, manifested by several mild neurologic changes, including abnormal pupillary reactions, hyperactive deep tendon reflexes, and bizarre motor or sensory changes. One patient with tabes dorsalis and one with dementia paralytica were also studied. Unfortunately, no patients with acute syphilitic meningitis, similar to the acute syndromes previously reported, came under treatment during the course of this analysis. At any rate, the different degrees of central nervous system involvement studied represented a series of cases more typically encountered in the penicillin treatment of neurosyphilis.

In Table III are listed the results of penicillin assay on spinal fluid, blood serum, and urine at intervals varying from 10 to 150 minutes following the intramuscular injection of 30,000 units. Several observations on the same patient indicate a serial study, in which the lumbar puncture needle was left in place, and spinal fluid specimens removed, at the intervals noted, simultaneously with the removal of blood. In several of the earlier cases studied, only single specimens were obtained. The results indicate that penicillin, within the limits of sensitivity of the assay test (0.125 unit per cubic centimeter), could *not* be detected in the spinal fluid in any of the patients. The reports of penicillin concentration in the spinal fluid of patients with meningitis were based on approximately the same unit sensitivity.

It has been previously emphasized that the usual methods employed in studying the absorption or excretion of penicillin are rather crude, since the selection of the procedure, in which the killing power of blood or other fluids containing penicillin is determined against the test organism, may give information of practical value in estimating the higher levels of the drug in that fluid but give no information regarding the biologic activity of material containing only traces of penicillin. Most investigators believe that this activity is very considerable and of great importance. It has, for example, been shown that penicillin induces a profound change in the morphology and growth of hemolytic streptococci in dilutions as high as one part in a billion, far beyond the zone of demonstrable bactericidal or bacteriostatic properties. It has yet to be determined whether these changes are of clinical significance.

The excellent results reported with the use of intramuscular penicillin therapy in patients with acute syphilitic meningitis[7] warrant further study to determine whether concentration is greater in these patients or whether penicillin in therapeutically adequate amounts, but not detectable by ordinary assay methods, is excreted in the spinal fluid. Since definite improvement has been shown to occur in many other patients with neurosyphilis[8, 9] after intramuscular penicillin, there is still the real possibility that the excretion of penicillin through the blood-spinal fluid barrier may not be necessary for its proper action in this disease. Certainly, the difficulties and occasional complications following the intrathecal administration of penicillin have resulted in increasing efforts to justify its administration by the intramuscular route in patients with neurosyphilis requiring prolonged and intensive therapy.

SUMMARY

Penicillin was administered intramuscularly in doses of 30,000 units to twelve subjects with neurosyphilis. Patients with asymptomatic neurosyphilis with varying degrees of spinal fluid cellular activity, meningovascular neurosyphilis, tabes dorsalis, and dementia paralytica were included in the study. Examination of the spinal fluid in these patients 10 to 150 minutes following injection did not reveal the presence of penicillin within the limits of the test employed (penicillin less than 0.125 unit per cubic centimeter).

Since beneficial results often follow the use of intramuscular penicillin in neurosyphilis, the drug may penetrate the spinal fluid in therapeutically effective amounts, not detectable by the methods employed, or penetration may not be necessary in a disease which is essentially vascular in nature.

Similar studies in patients with acute syphilitic meningitis may be of interest.

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# PLAGUE: TREATMENT OF EXPERIMENTAL ANIMALS WITH STREPTOMYCIN, SULFADIAZINE, AND SULFAPYRAZINE

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THE difficulties which attend the prevention and the successful treatment of plague stimulate continued studies of the therapeutic value of new drugs as they are developed. In 1944[1] we reported our results of treating experimental animals with sulfadiazine after the animals had developed plague. Hornibrook[2] conducted preliminary tests in this laboratory with streptomycin‡ in the treatment of white mice which were inoculated subcutaneously with *Pasteurella pestis* and concluded that it had therapeutic value under the circumstances of his experiments.

This report is a record of further investigations to evaluate streptomycin in the therapy of plague induced in mice and guinea pigs by inoculation and in guinea pigs which were infected by the bites of infectious fleas and to make comparisons of the results obtained by the use of this drug, and by sulfadiazine or sulfapyrazine.

## METHODS

Mice weighing approximately 20 grams each were selected from an inbred white Swiss colony. The guinea pigs were from dealer's stocks but had been under observation long enough to determine that they were healthy; those chosen weighed about 400 grams each. Plague was induced by inoculation in both mice and guinea pigs and by natural transmission through fleas to guinea pigs. The strain of *Past. pestis* in the inoculum was one which had been isolated during the year from the sputum of a patient who had pneumonic plague and had been maintained on blood tryptose agar. The virulence of *Past. pestis* for these animals had recently been proved, and it was freshly isolated from an infected mouse immediately previous to the commencement of each series of experiments. Streptomycin was dissolved in physiologic saline solution in concentrations which permitted a variation of the dose in a constant volume of 0.2 c.c. for mice and 1.0 c.c. for guinea pigs. The drug was given subcutaneously. The sodium salts of the sulfa drugs were similarly prepared and injected subcutaneously in 10 mg. doses in mice and 100 mg. doses in guinea pigs, as the initial dose to treatment with the respective sulfa compound. The sulfa compounds were suspended in acacia with double the dose of sodium bicarbonate and administered to the mice by mouth through a syringe. The guinea pigs received the compounds and soda by mouth in capsules or in a suspension in acacia.

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One-half of the inoculated animals were given the drugs, and the other one-half received the vehicle of the drug on test and served as controls. A few exceptions are noted. All the animals inoculated with *Past. pestis* throughout the experiments were examined at necropsy for gross pathologic and for bacteriologic evidence of plague.

### TESTS ON MICE

After a number of trial tests on groups of five or ten mice, six lots of forty each were inoculated, and treatment was commenced on one-half of each lot forty-eight hours later. The period of treatment extended for from five to eight days, and the animals were discharged and examined twenty-one days after inoculation.

Mice in three lots were given streptomycin in varying quantities per mouse, with varying amount and frequency of dosage. Those in the first lot received 1,800 units according to the following schedule: 400 units twice daily for two days; 300 units twice daily for two days; 200 units on one day; 100 units once daily on each of two days. Fifteen of the twenty mice survived. Those in the second lot received 1,200 units: 100 units twice daily for five days and once daily for two days. Eleven of twenty in the second lot survived. Two of the forty controls in these two lots survived.

The third lot was run as another experiment, and each mouse was given 2,200 units: 50 units every three hours for three days and 100 units four times daily for two days and once on the final day. Six of the twenty mice survived, and the twenty controls died. It was seen that under the 50 unit doses the mice were dying about as rapidly as the controls; hence the larger dose was adopted. The amount of each drug used and the day on which the mice in this lot, and subsequent lots, died are charted in Table I.

Mice in two lots received sulfadiazine. The first was given 70 mg. in 10 mg. doses twice daily for four days and once on the fifth day. Six of twenty treated and one control survived. Those in the second group had 75 mg. in 10 mg. doses twice daily for one day and 5 mg. twice daily for three days and once daily four more days. Seven survived among 110 treated, and the twenty controls died.

Mice in another lot received 50 mg. of sulfapyrazine in 5 mg. doses twice daily for four days and once daily for two days. Eight among nineteen survived, and the twenty controls died.

It was observed that from 25 to 40 per cent of different lots of control mice were dying before the end of the third day after inoculation and from 45 to 80 per cent on the fourth day. It appeared, therefore, that the infection had advanced in forty-eight hours to a degree which would not respond to therapy initiated on or after the third day, and a test was made to determine the rapidity with which a demonstrable bacteremia developed. Blood cultures were made from each of ten mice at intervals of twenty-four hours after the inoculation of a lot of forty. No positive cultures were obtained after the first twenty-four hours, but three were positive after forty-eight hours, five after seventy-two hours, and six after ninety-six hours. Among fifteen mice that

TABLE I. SHOWING DAY OF DEATHS AND SURVIVALS AMONG WHITE MICE TREATED WITH STREPTOMYCIN, SULFADIAZINE, AND SULFAPYRAZINE IN DIFFERENT AMOUNTS GIVEN FOR PERIODS OF SIX OR SEVEN DAYS AND STARTING FORTY-EIGHT OR THIRTY-TWO HOURS AFTER INOCULATION

| TREATMENT | NUMBER OF MICE | DEATHS—DAYS AFTER INOCULATION | | | | | | | | | | | | | | | | | | | NUMBER OF SURVIVALS | PER CENT OF SURVIVALS |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | | |
| Streptomycin, 1,800 units | 20 | | | | | | | | 1 | | 1 | | | | | 1 | | | 2 | | | | 15 | 75.0 |
| Controls | 20 | | | 6 | 7 | 4 | | | 1 | 1 | | | | | | | | | | | | | 1 | 5.0 |
| Streptomycin, 1,200 units | 20 | | | 2 | 1 | | | 2 | 2 | 2 | | | | | | | | | | | | | 11 | 55.0 |
| Controls | 20 | | | 6 | 2 | 2 | 3 | 4 | 1 | | | | | 1 | | | | | | | | | 1 | 5.0 |
| Streptomycin, 2,200 units | 20 | | | 4 | | 2 | 3 | | 1 | 1 | 1 | | | 1 | | | | | 1 | | | | 6 | 30.0 |
| Controls | 20 | | 2 | 1 | 10 | 3 | 2 | 1 | | | 1 | | | | | | | | | | | | 0 | 0.0 |
| Sulfadiazine, 70 mg. | 19 | | | 1 | 1 | 1 | 5 | 3 | 1 | | | | | | | | | | | | | | 6 | 31.5 |
| Controls | 20 | | | 1 | 3 | 4 | 2 | 3 | 3 | 3 | | | | | | | | | | | | | 1 | 5.0 |
| Sulfadiazine, 75 mg. | 19 | | 1 | 3 | | 1 | 1 | 2 | | 2 | 1 | | | | | | | 1 | | | | | 7 | 36.8 |
| Controls | 20 | | 1 | 4 | 7 | 3 | 3 | | 1 | 1 | | | | | | | | | | | | | 0 | 0.0 |
| Sulfapyrazine, 50 mg. | 19 | | | 4 | 2 | 1 | 1 | 3 | 2 | 2 | | | 1 | | 1 | | | | | | | | 2 | 10.5 |
| Controls | 20 | | 1 | 8 | 7 | 1 | 2 | 1 | | | | | | | | | | | | | | | 0 | 0.0 |
| Streptomycin, 2,600 units | 19 | | | | | | | 1 | | 1 | | | | 1 | | | | | | | | | 16 | 84.2 |
| Controls | 20 | | 1 | 8 | 4 | 4 | 1 | 1 | 1 | | | | | | | | | | | | | | 0 | 0.0 |
| Sulfadiazine, 65 mg. | 20 | | | | 1 | | | 1 | | | | | | | | | | | | | | | 18 | 90.0 |
| Sulfapyrazine, 65 mg. | 18 | | | | | | | | 1 | | | | | | | | | | | | | | 17 | 94.4 |
| Controls | 20 | | | 6 | 2 | 5 | 1 | 2 | 1 | 1 | | 1 | | | | | | | 1 | | | | 0 | 0.0 |

had negative blood cultures and survived the twenty-four or forty-eight hour bleeding, nine had positive cultures when bled seventy-two hours after inoculation.

Following these findings, 120 mice were inoculated, and thirty-one hours later treatment was started on sixty of these. Forty were carried as controls on those treated. Nineteen were given 2,600 units of streptomycin in 200 unit doses, once the first day, twice daily for five days, and once daily for two more days. Sixteen survived. Twenty had 10 mg. of sodium sulfadiazine on the first day, followed by 65 mg. of sulfadiazine on preceding days. A 10 mg. and a 5 mg. dose were given on the second day and 5 mg. twice daily for four days and once daily for two days. Sixteen of these mice survived.

Eighteen were similarly treated with sulfapyrazine, and seventeen survived. The forty controls on these three lots died.

Blood cultures were made on twenty others thirty-one hours after inoculation and two were positive.

While these tests were in progress, two groups of twenty uninoculated mice were given the same respective amounts and dosage of sulfadiazine and sulfapyrazine used in the tests. These animals were then sacrificed and examined for gross evidence of damage by the drugs. No pathologic changes were found. A similar absence of findings had been obtained previously after administration of streptomycin.

Many of the mice recovered under the treatment adopted with each drug. It seemed evident, however, that large numbers or many lots would be required to overcome the practical difficulties inherent in the use of these animals. They exhibit no clinical changes by which the infection can be detected readily, and bleeding them from the heart often causes irreparable damage. Furthermore, it is often impracticable to repeat all the conditions prevailing in tests on each of many lots. On the other hand, the guinea pig is very susceptible to plague, and one can easily obtain good clinical evidence of infection after both inoculation and flea transmission. It seemed desirable, therefore, to attempt to confirm in guinea pigs the results obtained in the mice before making any appraisals of the therapeutic value of the drug.

### TESTS ON INOCULATED GUINEA PIGS

The guinea pigs were tested in pairs. Treatment was given to the sicker of the two from forty-eight to seventy-two hours after inoculation, and the other animal served as a control. A rise in temperature above 39.3° C. and/or the development of a bubo in a lymph node adjacent to the site of the intracutaneous inoculation on the abdomen was regarded as evidence of infection. Animals were discharged after they had survived for twenty-one days after inoculation or for ten days after apparent recovery.

Five guinea pigs were each given from 20,000 to 26,000 units of streptomycin in 2,000 unit doses twice daily for six days and seemed to be recovering. Their temperatures had fallen to within a normal range and the buboes had decreased in size. Treatment was stopped. Forty-eight hours later all had either a rise in temperature or an increase in the size of the bubo. Two had developed a bacteremia. Treatment was repeated and during the following five to six days each received from 22,000 to 32,000 units and again appeared to have recovered. Ten days later, at necropsy, one animal had a soft purulent bubo from which *Past. pestis* was isolated. The others had some induration in and about the lymph nodes, but microorganisms were not obtained by cultural methods. The five controls died with plague within twelve days after inoculation.

Tests were made hourly on the blood of four guinea pigs which were given a single dose of 1,000 or 2,000 units of streptomycin in saline, or peanut oil, to determine the blood level of the drug. The method of Randall and associates[3] for the determination of amounts of penicillin in the blood was used. No streptomycin was found after three hours.

In a second experiment, nineteen pairs of guinea pigs were inoculated intracutaneously, and treatment with streptomycin was started from forty-eight to seventy-two hours later on one-half of them after fever and buboes had developed and a blood culture had been made on each. An initial dose of 3,000 units was followed by 2,000 unit doses repeated twice at intervals of two hours and thereafter at intervals of four, six, and eight hours through ninety-six hours. Treatment was then stopped, and three animals had a rise in temperature. Therefore all were given 2,000 units three times daily until apparent recovery from three to eight days later. The total amounts given individual animals varied from 39,000 to 72,000 units. All survived twenty-

five days from date of inoculation, although four had a bacteremia before treatment. Eleven showed no evidence of plague at necropsy. Seven had purulent buboes and one which had had a bacteremia had both a bubo and some lung lesions. *Past. pestis* was isolated from all the buboes and the lung lesion. Eighteen controls died with plague within nineteen days after inoculation. The survivor had no evidence of plague at necropsy on the twenty-fifth day, although it had had both fever and a bubo. The range of the amounts of the drug used primarily and with recurrences, and the results obtained in the animals, are charted in Table II. Examples of the different types of the temperature curves are shown in Fig. 1.

Five normal guinea pigs were each given streptomycin in amounts and dosage similar to those used in the test animals and appeared normal when examined at necropsy on the twenty-fifth day after their first dose.

Guinea pigs apparently tolerated the therapy well and recovered from plague under it even though they had developed a bacteremia before it was started. However, recovery did not occur as promptly as it did with the use of sulfadiazine in the tests previously reported.[1]

### TESTS ON GUINEA PIGS INFECTED BY FLEA TRANSMISSION

Plague is primarily a disease of rodents, and its mode of occurrence in Nature can be reproduced in the laboratory under controlled conditions by the bites of infected fleas. Thus, tests can be made of a therapy in the disease acquired in a natural manner and should afford a better evaluation of it and of the possibilities of its application in cases in human beings.

Four experiments were made with 106 guinea pigs which were infected with plague by the bites of fleas *(Xenopsylla cheopis)* that had been proved to be infectious, after feeding on a mouse artificially infected, with the strain of *Past. pestis* previously used. The general conditions, administration of the drugs, and division of the pairs for therapy and control were carried out as closely as possible to those previously used. Treatment was started when the clinical evidence of infection seemed assured, usually from three to five days after the flea bite. A blood culture was made of each animal at this time.

Fifteen animals were treated with streptomycin in 2,000 unit doses given three or four times daily, in accordance with their clinical responses, until apparent recovery. The first period of treatment extended for from eight to fourteen days, and the different individuals received from 46,000 to 92,000 units. Five had recurrences and were again treated for from two to eleven days, during which time they received from 4,000 to 57,000 units. Two had a second recurrence and received from 27,000 to 46,000 units over a period of from five to eight days. Fourteen survived for ten days after their clinical recovery and were then examined at necropsy. Two had soft buboes containing viable *Past. pestis.* One died of streptococcus pneumonia after having apparently recovered from plague during the first series of treatments. Six of the fourteen survivors had a bacteremia when treatment was started. There were twelve controls and all died with plague within ten days of the development of clinical findings.

TABLE II. SHOWING RESULTS OF TREATMENT OF GUINEA PIGS WITH STREPTOMYCIN AFTER PLAGUE HAD BEEN INDUCED BY INTRACUTANEOUS INOCULATION OF PAST. PESTIS (AMOUNTS OF DRUG RECORDED IN 1000 UNITS)

| TOTAL | NUMBER OF GUINEA PIGS | | | | REMARKS | AMOUNT OF DRUG IN FIRST TREATMENT | NUMBER OF GUINEA PIGS WITH RECURRENCE | AMOUNT OF DRUG IN SECOND TREATMENT | TOTAL AMOUNT OF DRUG |
|---|---|---|---|---|---|---|---|---|---|
| | WITH BACTEREMIA* | SURVIVED | DIED | WITH PLAGUE AT NECROPSY | | | | | |
| 5 treated with streptomycin | 0 | 5 | 0 | 1 | Purulent bubo | 20-26 | 5 | 22-32 | 44-57 |
| 5 controls | 0 | 0 | 5 | 5 | | | | | |
| 19 treated with streptomycin | 4 | 19 | 0 | 8 | Purulent bubo in seven; purulent bubo and lung lesions in one | 31-58 | 12 | 14-22 | 39-72 |
| 19 controls | 1 | 1 | 18 | 18 | | | | | |

*Bacteremia determined by culture of heart blood before treatment.

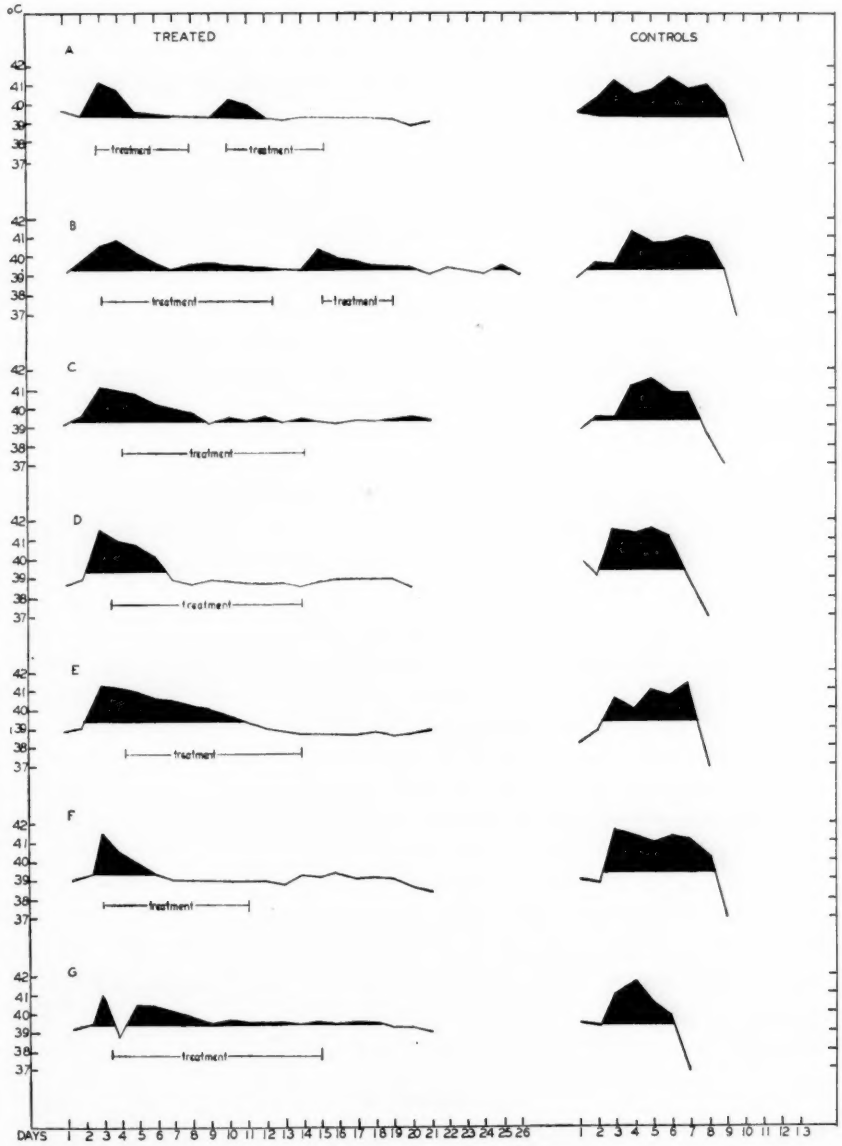


Fig. 1.—Types of fever charts of guinea pigs infected with plague and treated with streptomycin; sulfadiazine; sulfapyazine; streptomycin and sulfadiazine; and of untreated controls. (Fever charts were made of every guinea pig.)

*Treated: A, B, C,* Curves of 87 per cent streptomycin; *B,* curves of 60 per cent sulfapyrazine; *D, E,* curves of 93 per cent sulfadiazine; *E,* curves of 30 per cent sulfapyrazine; *F,* curves of 50 per cent sulfadiazine and sulfapyrazine; *G,* curves of five very sick with bacteremia, three treated with streptomycin, one with sulfapyrazine, one with streptomycin and sulfadiazine.

*Controls: A* to *G,* Types of various curves which occurred.

Fifteen were given sulfadiazine in 100 mg. doses once or twice daily for from six to fifteen days, after which no further treatment was indicated. The total amounts given individuals varied from 500 to 3,700 mg. All received an initial dose of 100 mg. of the sodium salt. Fourteen survived for from twenty to thirty-one days and showed no evidence of plague at necropsy. One died of plague on the second day of its treatment. This animal and six others had a bacteremia before treatment was commenced. There were fourteen controls and thirteen died within fifteen days after infection. The survivor did not have plague at necropsy on the twenty-fourth day, although it had previously had a large soft bubo.

Sixteen were treated with sulfapyrazine, and individual animals received from 500 to 8,000 mg. in 100 mg. doses given once or twice daily for from four to eighteen days. All were given an initial dose of the sodium salt, and seven received from 100 to 200 mg. additionally. Ten survived for from twenty to thirty-one days; four of these had buboes containing viable *Past. pestis* at necropsy. Five died of plague between the fourth and tenth day and one on the eighteenth day with extensive lung lesions, after having apparently recovered. Six of the lot had a bacteremia before they were treated. Fifteen of the sixteen controls died of plague between the third and sixteenth day after infection. One survived twenty-five days and did not have plague at necropsy although it had clinical findings previously.

Ten were treated with both streptomycin and sulfadiazine. The dosage was the same as that used with the single respective drug. Individual animals received amounts of from 18,000 to 26,000 units of streptomycin and from 1,000 to 2,000 mg. of sulfadiazine during periods of from five to eleven days. Two animals had a clinical recurrence and were given, respectively, 900 and 2,400 mg. of sulfadiazine in the course of from two to seven days. All the animals survived for from twenty to twenty-nine days, and eight did not have plague when examined at necropsy. Two had soft buboes containing viable *Past. pestis*. Three had a bacteremia before treatment. There were eight controls and seven died with plague from four to twelve days after infection. One survived twenty days and at necropsy had a soft bubo containing viable *Past. pestis*. The number of animals treated, the amounts of the drugs administered, the recurrences, and the survivals or deaths of animals are charted in Table III. Examples of the temperature curves are shown in Fig. 1.

## COMMENT

These experiments indicate that streptomycin has definite value in the treatment of white mice and guinea pigs after they have been infected with plague. It seems, however, to have less value in the treatment of mice which have developed a bacteremia than in the treatment of guinea pigs. The amounts and dosage of the drug seemed to be well tolerated by the animals for the periods of treatment. Among both the inoculated and the naturally infected guinea pigs there were a number of clinical recurrences after the animal had apparently recovered. It is possible that the recurrences may be corrected by the adoption of different methods of administration or of amounts

TABLE III. SHOWING RESULTS OF TREATMENT OF GUINEA PIGS WITH (A) STREPTOMYCIN, (B) SULFADIAZINE, (C) SULFAPYRAZINE, AND (D) STREPTOMYCIN PLUS SULFADIAZINE AFTER PLAGUE HAD BEEN INDUCED BY BITES OF INFECTIOUS FLEAS (AMOUNTS OF STREPTOMYCIN RECORDED IN 1,000 UNITS; SULFA DRUGS, IN 1,000 MG.)

| NUMBER OF GUINEA PIGS | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| TOTAL | WITH BACTERIEMIA* | SURVIVED | DIED | WITH PLAGUE AT NECROPSY | REMARKS | AMOUNT OF DRUG IN FIRST TREATMENT | GUINEA PIGS WITH A RECURRENCE | AMOUNT OF DRUG IN SECOND TREATMENT | GUINEA PIGS WITH A RECURRENCE | AMOUNT OF DRUG IN THIRD TREATMENT | TOTAL AMOUNT OF DRUG |
| 15 treated with streptomycin | 6 | 18-43 days<br>14 | (26th day)<br>1 | 2 | One pig died on twenty-sixth day with streptococcic pneumonia | 46-92 | 5 | 4-57 | 2 | 27-46 | 48-125 |
| 12 controls | 6 | 0 | 12 | 12 | | | | | | | |
| 15 treated with sulfadiazine | 7 | 20-31 days<br>14 | (5th day)<br>1 | 1 | One died on second day of treatment, fifth day after inoculation | 0.5-3.7 | 0 | | | | 0.5-3.7 |
| 14 controls | 5 | 24 days<br>1 | 13 | 13 | One developed bubo and fever but recovered | | | | | | |
| 16 treated with sulfapyrazine | 6 | 20-31 days<br>10 | 6 | 10 | Four had purulent buboes only but with viable P. pestis | 0.5-2.85 | 6 | 0.9-2.5 | 0 | | 0.5-8.0 |
| 16 controls | 7 | 25 days<br>1 | 15 | 15 | One developed bubo and fever but recovered | | | | | | |
| 10 treated with<br>(a) streptomycin and<br>(b) sulfadiazine | 3 | 20-29 days<br>10 | 0 | 2 | Two had soft purulent buboes only but with viable P. pestis | (a) 18-26<br>(b) 1-2 | 2 | (b)<br>0.9-2.4 | 0 | | (a) 18-26<br>(b) 1.0-4.4 |
| 8 controls | 1 | 20 days<br>1 | 7 | 8 | One had soft purulent bubo only but with viable P. pestis | | | | | | |

*Bacteremia determined by culture of heart blood before treatment.

and timing of the dosage, since it appears that the drug remains in the blood stream for a very short period. In several instances, a soft purulent bubo remained after the apparent recovery of a guinea pig treated by streptomycin or by another of the drugs. In the light of experience with plague in rodents, it may be assumed that most of such animals had recovered and that the bubo would have healed and the microorganisms would have been eliminated without further therapy.

The results of the treatment of guinea pigs with sulfadiazine are confirmatory of those previously reported by us. Although sulfadiazine appeared to bring about recovery more promptly than did streptomycin, it does not seem that comparison of the two is justified because of the reasons previously expressed.

The use of a combination of streptomycin and sulfadiazine in treatment does not seem to add to the effectiveness of either. Sulfapyrazine offers no advantages over sulfadiazine.

The difficulties of appraising the progress of plague in white mice, and the rapidity with which they develop bacteremia and die, necessitates the use of many lots of animals with the probabilities of the introduction of different conditions while making an adequate test of a therapy. For these reasons it is believed that remedies to be used in plague should be tested on other animals.

## SUMMARY

1. Three hundred and thirty-four white mice were inoculated with plague, and one-half were treated in groups with different amounts and varying dosage of either streptomycin, sulfadiazine, or sulfapyrazine. The other one-half were used as controls. Under the treatment schedules finally adopted, from sixteen to eighteen survived among each lot of from eighteen to twenty, while all the corresponding controls died with plague.

2. Forty-eight guinea pigs were likewise inoculated, and one-half were treated with streptomycin in two groups after clinical evidence of infection had developed. All survived, but eight had residual buboes at necropsy. Twenty-three controls died with plague.

3. One hundred six guinea pigs were infected through flea transmission. After the disease was evident clinically, fifteen were treated with streptomycin and fourteen survived; fifteen received sulfadiazine and fourteen survived; ten received both these drugs and all survived; sixteen received sulfapyrazine and ten survived. Six among all the survivors had residual buboes at necropsy. Three survived among fifty controls divided among the four lots treated.

4. Clinical recurrences appeared frequently under the treatment schedules used with streptomycin and sulfapyrazine but not with that of sulfadiazine.

We wish to thank Cleon W. Abbott, Medical Technician, for the technical assistance given.

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# THE EFFECT OF GOLD CHLORIDE ON PLASMA ASCORBIC ACID IN THE RAT

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THE complications which so frequently attend the clinical use of gold compounds suggest the value of investigating the effect of such substances on experimental animals. In the present series of experiments, gold was employed in one of its simplest forms, yellow gold chloride. It was administered by intraperitoneal injection of 0.5 per cent aqueous solution. The rats used were of the Long-Evans strain and were fed a modified Steenbock diet.

## TOLERANCE TO GOLD CHLORIDE

To determine the tolerance of the rat to yellow gold chloride, graded single doses were given to a series of rats and the survival time was noted. The results are recorded in Table I. To facilitate comparison with other gold salts, the dosage is given in terms of milligrams of gold per kilogram of body weight of the animal rather than in milligrams of gold chloride.

Single doses smaller than those listed in Table I were not lethal. With a dosage of 20 mg. of gold per kilogram, a rat weighing 215 grams tolerated daily injections for five days, dying on the sixth day. These results may be compared with those of Cortell and Richards,[1] who employed intramuscular injection in rats. They found a minimum lethal dose of approximately 13 mg. of gold per kilogram for gold sodium thiosulfate, while 37 mg. of gold per kilogram were tolerated if administered in the form of gold sodium thiomalate.

On the basis of these results, it was concluded that a dosage of 15 mg. of gold per kilogram would be suitable for the repeated intraperitoneal administration of gold chloride to rats. In the course of the chronic experiments, it was found that an increase in tolerance had developed. This is illustrated by a rat which received nine injections of 15 mg. of gold per kilogram, five of 20 mg. per kilogram, and, finally, five of 25 mg. per kilogram before succumbing. Of two other rats, one survived eleven and the other thirteen doses of 20 mg. of gold per kilogram, after having previously received fourteen and nine doses, respectively, of 15 mg. per kilogram. This increase in tolerance to gold chloride is similar to the development of tolerance to gold sodium thiosulfate reported by Cortell and Richards.[1]

The gold-treated animals lost weight during the first three days of treatment, the average loss being 10 grams per animal. Following this loss, however, they again started gaining weight, the average increase being 2.0 grams per day for the rats receiving 15 mg. of gold per kilogram, compared with 2.4 grams per day for the controls. Increasing the dosage of gold decreased the

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TABLE I. SURVIVAL TIME AFTER SINGLE INTRAPERITONEAL DOSES OF GOLD CHLORIDE

| WEIGHT OF RAT (GM.) | DOSE (MG. GOLD KG.) | SURVIVAL TIME (HR.) |
|---|---|---|
| 125 | 50 | 2 |
| 175 | 40 | 5 |
| 202 | 30 | 7 |
| 186 | 28 | 8 |
| 178 | 25 | 15 |
| 190 | 25 | 40 |
| 187 | 23 | 51 |

growth rate. The figures given do not include the terminal period of existence, during which abdominal distention following renal failure frequently occurs, together with a marked loss in weight.

### PLASMA ASCORBIC ACID OF GOLD-TREATED RATS

The effect of gold chloride administration on the level of ascorbic acid in the plasma was determined in two series of rats. In the first experiment, the ascorbic acid was determined from samples of blood drawn two days after the last injection of gold chloride. The determinations of ascorbic acid were made by the method of Farmer and Abt[2]; the data are given in Table II.

Although the results of this experiment definitely indicate a decrease in the ascorbic acid content of the plasma of the rats receiving gold chloride, it was thought advisable to repeat the experiment, obtaining initial ascorbic acid values for some of the experimental animals. The major portion of this experiment was terminated at the end of two weeks, but two of the experimental animals were kept until the end of five weeks. During the first two weeks, each rat received nine intraperitoneal injections of 15 mg. of gold per kilogram. The two animals kept under treatment during the following three weeks received, in addition, five injections of 15 mg. per kilogram and eight of 20 mg. per kilogram, the total administered during the five weeks being 370 mg. of gold per kilogram. The decrease in plasma ascorbic acid in the gold-treated rats is shown by Table III. Death intervened due to the effect of the gold on the kidneys before definite scorbutic symptoms were observable.

Similar results were obtained in preliminary experiments on guinea pigs. In two guinea pigs receiving seven injections of 10 mg. of gold per kilogram during a ten-day interval, the plasma ascorbic acid dropped from 0.6 and 0.5

TABLE II. PLASMA ASCORBIC ACID OF GOLD-TREATED RATS; FIRST EXPERIMENT: GOLD CHLORIDE ADMINISTERED DAILY; PLASMA ASCORBIC ACID DETERMINED TWO DAYS AFTER LAST DOSE OF GOLD CHLORIDE

| TREATMENT | TOTAL GOLD (MG. KG.) | FINAL PLASMA ASCORBIC ACID (MG. %) |
|---|---|---|
| Average of 11 controls (range of ascorbic acid, 0.78 –1.32 mg. %) | 0 | 1.04 |
| Seventeen doses of 2.0 mg./kg. | 34 | 0.82 |
| Five doses of 0.6 mg./kg. and eleven doses of 4.0 mg./kg. | 47 | 0.82 |
| Six doses of 1.3 mg./kg. and eleven doses of 9.0 mg./kg. | 107 | 0.66 |
| Seven doses of 7.5 mg./kg. | 53 | 0.49 |
| Seven doses of 10.0 mg./kg. | 70 | 0.49 |

TABLE III. PLASMA ASCORBIC ACID OF GOLD-TREATED RATS; SECOND EXPERIMENT

| | PLASMA ASCORBIC ACID (MG. %) | | |
|---|---|---|---|
| | INITIAL | 2 WK. | 5 WK. |
| Controls | 1.32 | 0.67 | 0.86 |
| | 0.78 | 0.57 | 0.75 |
| Gold-treated rats | 1.16 | 0.26 | 0.13 |
| | -- | 0.38 | 0.28 |
| | 1.09 | 0.33 | |
| | 1.01 | 0.46 | |
| | -- | 0.41 | |
| | 1.01 | 0.27 | |
| | -- | 0.39 | |

During the first two weeks nine doses of 15 mg. gold per kilogram were given; the two animals which were kept for five weeks received five additional doses of 15 mg. gold per kilogram and eight of 20 mg. gold per kilogram.

mg. per cent to 0.0 mg. per cent. The control showed 0.8 mg. per cent of plasma ascorbic acid at the beginning of the experiment and 0.5 mg. per cent after ten days. Data from such a small number of animals are significant only in that they indicate that the reaction of the guinea pig to gold chloride is similar to that of the rat.

## COMMENT

The depletion of the plasma ascorbic acid of the rat by the administration of gold chloride is especially interesting since the rat is able to *synthesize* ascorbic acid. If the rat alone suffered this depletion upon gold treatment, there would be a possibility that the gold affected the process of synthesis. The experiments on guinea pigs, however, indicate that in these animals also the administration of gold chloride is followed by decrease in plasma ascorbic acid. A decrease in vitamin C in certain tissues, notably adrenal, lung, and duodenum, has been reported by Sande[3] after administration of Sanocrysin.

The mechanism by means of which the depletion takes place is unknown. It is possible that the gold chloride may oxidize the ascorbic acid directly, a reaction which proceeds with facility in the test tube. If this reaction takes place in the body, it might be expected to lead to an accumulation of metallic gold in the cells which contain active ascorbic acid. Such deposits might well be dense enough, on occasion, to be visible under the microscope. Histologic studies of the distribution of gold in the tissues, to be reported in detail later, do not reveal a parallelism between the distribution of gold and of ascorbic acid. The depletion of ascorbic acid may be part of a general toxic reaction rather than a specific effect of gold chloride. In either case, the recognition of its occurrence after gold chloride administration is of value.

## SUMMARY

The plasma ascorbic acid of the rat is markedly decreased by the intraperitoneal administration of gold chloride. Repeated injections of gold result in the development of increased tolerance and a diminished growth rate.

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# FURTHER STUDIES ON THE ANTIBACTERIAL ACTION OF 3′, 5′-DIBROMOSULFANILANILIDE

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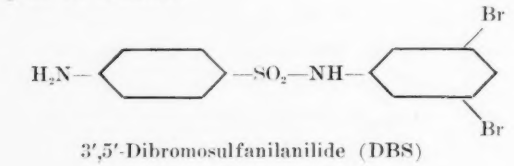
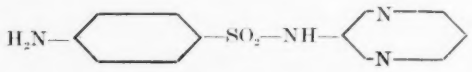
A PREVIOUS communication from these laboratories presented the antibacterial effects of a group of benzenesulfonic acid derivatives.[1] In general, the most active members of this group were 3′,5′-dibromosulfanilanilide and the corresponding dichloro derivative. Not only were these compounds found to be highly active against such pathogens as types I, II, and III pneumococci, *Streptococcus pyogenes* (C203), *Staphylococcus aureus,* Groups I and IIa meningococci, *Clostridium welchii, Clostridium tetani, Shigella paradysenteriae* (Hiss), and *Vibrio cholerae,* but they proved to be as effective against a sulfonamide-resistant strain of the gonococcus as against several sulfonamide-susceptible strains. In addition to the above attributes, the compounds were found to be unaffected by the presence of p-aminobenzoic acid when tested against *Str. pyogenes.*

Inasmuch as these compounds exhibited several properties not associated with the common sulfonamides (sulfanilamide, sulfadiazine, sulfathiazole, etc.), an extensive pharmacologic study was made of 3′,5′-dibromosulfanilanilide (hereinafter referred to as DBS). The structural relationship of DBS to sulfanilamide and the two substituted heterocyclic sulfonamides used in the present study appear as follows:

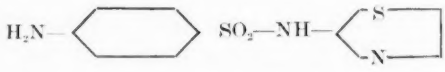



3′,5′-Dibromosulfanilanilide (DBS)

Sulfanilamide




Sulfadiazine




Sulfathiazole

The physical properties, methods of chemical determination, acute and chronic toxicities, absorption, metabolism, and excretion of DBS were investigated, and the results will be reported by McChesney, Goetchius, and Lawrence.[2] It was found that the toxicity of DBS was of the same order as that of sulfathiazole, which is slightly more toxic than sulfanilamide. Only minute amounts were found to be excreted in the free form in the urine of dogs and rats. In rabbits, however, the larger proportion of the dose was found in the free form in the urine. In man, the drug was excreted in a con-

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jugated form which lost some of the bactericidal potency of the original compound. Rapid and satisfactory absorption occurred in rats, inasmuch as the drug was readily demonstrated in the blood following oral administration. In man, only small amounts of free DBS appeared early in the blood, but rather large amounts of the conjugated form (from 4 to 5 mg. per cent) appeared several hours later.

These encouraging pharmacologic findings prompted a study of the antibacterial action of DBS upon several other pathogenic microorganisms. The results of this investigation are presented herewith.

## ORGANISMS TESTED AND METHODS OF STUDY

The general method of study consisted of making an initial dilution of 1:1000 DBS in an appropriate broth medium and of preparing serial dilutions from this in the same broth. Due to its relative insolubility in water, DBS was first dissolved in a small amount of alcohol preparatory to the initial dilution. Control tests showed this amount of alcohol to have no inhibitory effects upon the growth of the test organisms. For purposes of comparison, sulfadiazine and sulfathiazole were dissolved directly in broth and treated in a similar manner. The tubes, containing a total of 5 c.c. (10 c.c. in the case of Brewer's fluid thioglycollate medium), were autoclaved at 10 pounds for ten minutes. After cooling, each tube of a dilution series was inoculated with a 4 mm. loopful of a twenty-four hour broth culture of one of the test organisms. The tubes were incubated at 37° C. and examined for growth after twenty-four hours. Tubes showing no visible growth or growth less than one-half of that in the drug-free control were considered to contain sufficient drug to cause bacteriostasis of the test organism. Those tubes showing no growth after seventy-two hours' incubation were subcultured by transferring 3 loopfuls to fresh medium lacking the drug. Failure of growth to appear in the subculture tube after seventy-two hours' incubation at 37° C. was taken as evidence that the organisms were destroyed in the original drug-organism-broth mixture.

TABLE I. HIGHEST DILUTION OF COMPOUND EXHIBITING ANTIBACTERIAL ACTION

| ORGANISM | BROTH MEDIUM | DBS | | SULFADIAZINE | | SULFATHIAZOLE | |
|---|---|---|---|---|---|---|---|
| | | BS | BC | BS | BC | BS | BC |
| Gaffkya tetragena (RG) | Beef extract | 32,000 | 32,000 | <1,000 | <1,000 | <1,000 | <1,000 |
| Gaffkya tetragena (MB) | | 64,000 | 32,000 | 2,000 | <1,000 | 16,000 | <1,000 |
| Streptococcus agalactiae | Veal infusion + | 64,000 | 32,000 | 1,000 | <1,000 | <1,000 | <1,000 |
| Listerella monocytogenes | 0.15% dextrose | 128,000 | 16,000 | <1,000 | <1,000 | <1,000 | <1,000 |
| Streptococcus viridans | Veal infusion dex- | 16,000 | 8,000 | <1,000 | <1,000 | <1,000 | <1,000 |
| Hemophilus ducreyi | trose + 0.1% horse serum | 512,000 | 32,000 | 64,000 | <1,000 | 256,000 | <1,000 |
| Brucella abortus | Tryptose-phosphate | 64,000 | 16,000 | 32,000 | <1,000 | 4,000 | <1,000 |
| Brucella melitensis | + 0.1% agar | 128,000 | 32,000 | 32,000 | <1,000 | 8,000 | <1,000 |
| Brucella suis | | 256,000 | 16,000 | 32,000 | <1,000 | 16,000 | <1,000 |
| Actinomyces bovis (anaerobic) | Brewer's fluid thioglycollate | 128,000 | 16,000 | 2,000 | <1,000 | 64,000 | <1,000 |

BS, Bacteriostatic.
BC, Bactericidal.
<1000, Concentrations greater than 1:1000 not tested.

The organisms tested, the medium employed in each case, and the extent of antibacterial action are presented in Table I.

## RESULTS AND DISCUSSION

It may be noted from the data given in Table I that DBS exerts a far greater antibacterial action upon all the test organisms than does sulfadiazine or sulfathiazole. The latter two failed to show any bactericidal action in a 0.1 per cent concentration, whereas DBS was bactericidal for all the organisms.

The history of the two strains of *Gaffkya tetragena* is of more than passing interest. Both of these strains were recent isolations from individuals suffering from infection. Strain MB was isolated from a draining sinus of the hip, while strain RG was isolated from the blood stream of a patient with septicemia. Laboratory tests on strain RG produced some unusual results. This organism was completely unaffected by sulfadiazine, sulfathiazole, or sulfamerazine, even in a 1 per cent concentration of the drugs. Furthermore, 5,000 units per cubic centimeter of penicillin were required to inhibit its growth. This is another instance in which a sulfonamide-resistant organism (also penicillin-resistant) is susceptible to the antibacterial action of DBS.

The treatment with sulfonamides and penicillin of subacute bacterial endocarditis caused by *Streptococcus viridans* has met with varying degrees of success.[3, 4] The sulfonamides have been employed successfully in the treatment of experimental infections caused by *Listerella monocytogenes*[5] and by *Hemophilus ducreyi*.[6, 7]

In the treatment of actinomycosis and brucellosis, sulfonamides and penicillin are of questionable value. While these agents did exert some action against *Actinomyces bovis* in vitro, Keeney and associates[8, 9] caution that the action of sulfonamides on *A. bovis* is not spectacular and that expectancy of cure from their use in human actinomycosis should be regarded with conservatism.

In reviewing the English literature on the sulfonamide therapy of brucellosis, Urschel[10] concluded that the sulfonamides are of doubtful value in the therapy of brucellosis, inasmuch as the percentage of successful therapy was no higher than might be expected among untreated patients with this disease.

From this brief summary of the status of the chemotherapy of infections caused by the organisms listed, it is apparent that in most cases the available sulfonamides fall far short of being satisfactory. A comparison has been made between DBS and two commonly employed sulfonamides. Whether or not sulfathiazole or sulfadiazine had been effective in any of the disease-conditions discussed, DBS has a superior in vitro activity against the organisms responsible for these infections, an observation which suggests its trial in vivo.

## SUMMARY

1. The activity of 3′,5′-dibromosulfanilanilide has been compared to that of sulfadiazine and sulfathiazole against such organisms as ***G. tetragena, Streptococcus agalactiae, Str. viridans, L. monocytogenes, H. ducreyi, Brucella abortus, Brucella melitensis, Brucella suis,*** and ***A. bovis.***

2. A greater bacteriostatic effect and a far greater bactericidal effect was exerted by 3′,5′-dibromosulfanilanilide than by sulfadiazine or sulfathiazole against these organisms.

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# THE APPLICATION OF THE "LYOPHILE TECHNIQUE" TO BIOLOGIC PRODUCTS

## I. Sera, Antisera, and Antibody Globulins.

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IN 1931, this laboratory began work on the development of the "lyophile technique." Since that time, the process has been applied to a variety of biologic products. It is the purpose of this paper to summarize the results of this experience and to point out some of the practical limitations of the process. This phase of the subject has not received the same adequate consideration as the study of the fundamental principles underlying this method of drying and of designing a satisfactory apparatus to carry on the process.

A desiccation process, to be of practical value, must meet certain critical requirements. When applied to biologically active substances, the dehydrated product (1) must show no substantial loss of potency as a result of the desiccation process; (2) must show a permanency of preservation not only under favorable, but also under adverse, temperature conditions similar to those encountered in tropical climates; and (3) should show complete and reasonably quick solubility so that the average practitioner can administer it to the patient with the least effort and expenditure of time to himself and with the least annoyance and anxiety of waiting to the patient.

The biologic products which have been desiccated by the "lyophile technique" and which have been studied by us include experimental lots as well as products prepared under actual routine commercial conditions. Unless otherwise specified, the residual moisture content of the products was held to a maximum of 1.0 per cent or less. Examples are given from various classes of products studied and the degree to which they fulfill the three requirements previously mentioned is indicated. From these examples, we are able to make a few generalized statements relative to the application of the "lyophile technique" to biologic products and to point out its advantages and practical limitations.

Products which have been processed and studied may be classified conveniently as follows: (1) antisera (including normal guinea pig serum and human plasma); (2) antibody globulins; (3) viruses and virus vaccines; (4) live bacterial suspensions.

Antisera and antibody globulins only will be discussed in this communication.

### SERA AND ANTISERA

Many investigators had previously reported on the keeping qualities of dry immunologically active substances, including antisera. Some failed to specify

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the temperature under which they were kept, and others merely stated that the particular substance in question retained its properties when kept under the favorable temperature condition of the icebox. The relationship of the moisture content of the product under preservation to keeping qualities rarely was considered and seldom was discussed.

In order to obtain this desirable information, we investigated the influence of three factors, time, temperature, and residual moisture content, on the potency of antitoxic and antibacterial sera. Diphtheria antitoxic serum was studied more thoroughly than some of the others because its potency can be determined with a fair degree of accuracy by means of the in vitro flocculation test and by the in vivo $L^+$ test. The results obtained on this product are given in detail, presumption being that the conclusions derived from these results apply in a general way to the other antitoxic sera studied.

The serum was placed in 20 c.c. amounts into 50 c.c. containers and was frozen at –78° C., after which it was dehydrated on a manifold at the temperature of the surrounding air. The period of desiccation was varied purposely so that the end products would contain varying amounts of moisture. This was done by removing some of the containers from the manifold after twelve, sixteen, twenty, twenty-four and forty-eight hours of desiccation. Sample containers from each group were tested for moisture content, Lf (flocculation units), Kf (time of flocculation), and for antitoxic units by the standard guinea pig test. The results are shown in Table I.

TABLE I. "LYOPHILE" DIPHTHERIA ANTITOXIC SERUM (RESTORED IMMEDIATELY AFTER DESICCATION)

| DESICCATION PERIOD (HR.) | GROUP | MOISTURE CONTENT (PER CENT) | LF UNITS | KF (MIN.) | ANTITOXIC UNITS GUINEA PIG TEST |
|---|---|---|---|---|---|
| 12 | 1 | 15.6 | 750 | 35 | 700 |
| 16 | 2 | 2.6 | 750 | 37 | 600 |
| 20 | 3 | 1.9 | 750 | 31 | 600 |
| 24 | 4 | 1.4 | 750 | 25 | 600 |
| 48 | 5 | 1.0 | 750 | 26 | 700 |
| Original liquid serum untreated | | | 750 | 26 | 700 |

It is apparent that the Lf, Kf, and the antitoxic potency of the serum had not changed significantly as a result of the desiccation process.

A dozen containers (stoppered under vacuum) from each group were then stored at 5° C. and at 40° C. A sample container from each group was taken at the end of one- three, six, and thirteen months and the serum restored with the proper amount of distilled water and tested for potency ($L^+$ units), Lf units, and Kf. In order to save space, the protocols of the one-, three-, and six-month aging tests are omitted. The results of the same serum groups tested after thirteen months are given in Table II.

The aging tests bring out certain interesting facts. After one month of storage at 40° C., the serum in Group 1 was no longer completely soluble and after three months' storage, was completely insoluble. The corresponding sample kept at 5° C. for three months dissolved completely and showed no loss in potency. This demonstrated very clearly that a high moisture content in

TABLE II. "LYOPHILE" DIPHTHERIA ANTITOXIC SERUM (STORED AT 5° C. AND AT 40° C. FOR THIRTEEN MONTHS)

| | | 5° C. | | | 40° C. | | |
|---|---|---|---|---|---|---|---|
| GROUP | MOISTURE CONTENT (PER CENT) | L+ UNITS | LF UNITS | KF (MIN.) | L+ UNITS | LF UNITS | KF (MIN.) |
| 1 | 15.6 | 700 | 750 | 60 | Insoluble; jellied | | |
| 2 | 2.6 | 600 | 750 | 38 | 700+ | 750 | > 13 Hr. |
| 3 | 1.9 | 700 | 750 | 39 | 700 | 750 | 13 Hr. |
| 4 | 1.4 | 600+ | 750 | 40 | 600 | 750 | 13 Hr. |
| 5 | 1.0 | 700 | 750 | 42 | 600 | 750 | 13 Hr. |

an apparently dry-looking serum leads to rapid deterioration after an exposure to a temperature of 40° C. for one month.

The serum in Groups 2 to 5 inclusive, containing from 2.6 to 1.0 per cent moisture, showed no significant change in potency after thirteen months' exposure to 40° C. That the serum in these groups did undergo some change, however, is revealed by the longer time it took to dissolve the dry serum completely and by the marked increase in the Kf or time of flocculation. The restored samples of serum showed a faint haze compared with those samples stored at 5° C. and similarly restored.

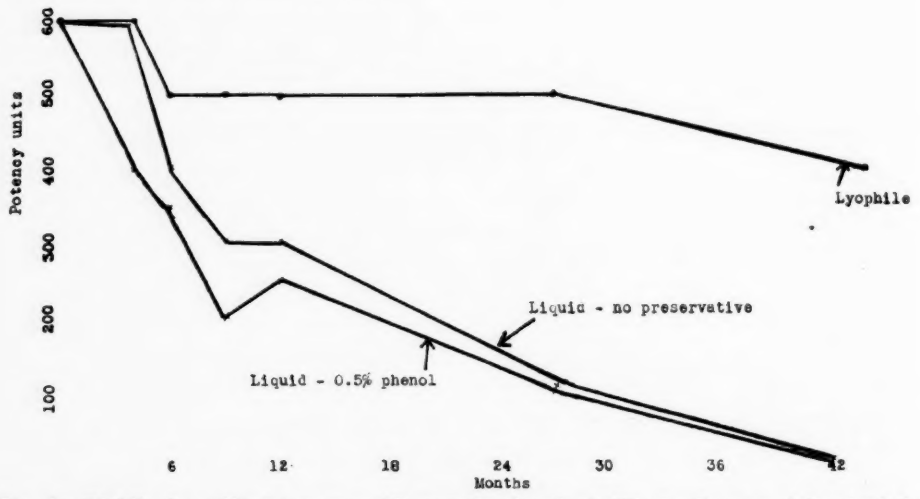


Fig. 1.—Diphtheria antitoxic serum. All samples stored at 37° C. Potency determined by animal tests.

In another experiment diphtheria antitoxic serum was divided into three parts: (1) without preservative, (2) with 0.5 per cent phenol, (3) without preservative. All three portions were filled into all-glass containers; Parts 1 and 2 were left in the liquid state and Part 3 was lyophilized and sealed under vacuum. All containers were stored at 37° C. and the potency of the serum was tested at various intervals, using the standard animal test. The results are graphically represented in Fig. 1. At this temperature, the longer preservation or retention of potency of the "lyophile" material certainly is very striking.

The influence of time, temperature, and residual moisture content on the potency of antibacterial sera and particularly antimeningococcic serum was also investigated. The "lyophile" serum and the original liquid serum were filled into suitable containers and stored at 5° C., room temperature (from 20 to 32° C.), and 37° C. Both liquid and "lyophile" sera were tested for agglutinin titer after two, three, eight, and 15 months of storage. The results obtained may be briefly summarized as follows.

After a storage period of two months at room temperature and at 37° C., the liquid sample had lost its agglutinin titer completely, whereas after storage for fifteen months at 37° C., the "lyophile" sample still retained a very substantial portion of its agglutinin titer which was about equal to that retained by the liquid sample kept at 5° C. for the same period.

The difference in physical appearance of the various samples also was impressive. All the liquid samples, regardless of the temperature of storage, showed marked physical changes manifested by a change in color and by the presence of considerable flocculent material. The "lyophile" serum samples all dissolved easily and completely and yielded a clear yellow fluid resembling in appearance the original liquid serum before it was aged.

## ANTIBODY GLOBULINS

To minimize the incidence of serum reactions, it has been the practice for many years to purify and concentrate therapeutic sera. The work of McFarlane[1, 2] and Pedersen[3] points to the possibility that changes in the molecular and other properties of the serum globulins are produced by the process of purification. This and the practice of including a given dosage in a minimal volume introduce factors which influence markedly the hydrophilic properties of dry globulins.

Antitoxic globulin (diphtheria, tetanus, perfringens) preparations may be readily converted to the "lyophile" state. The resulting dry mass presents a much more compact and less friable structure than that obtained with unpurified sera. Apparently the interstices between the protein fibrous structure are not as numerous and the mass is, therefore, not as porous. On the addition of water, the diffusion of the solvent throughout the mass is relatively slow. In addition, the hydrophilic property of the globulins is less pronounced than that of whole serum. As a result, the time of dissolution or restoration of "lyophile" antitoxic globulins is greatly prolonged.

Although the dry globulins dissolve completely and the potency of the restored material is essentially that of the unprocessed material, the excessively long time required for dissolution makes their clinical use almost impractical.

The biologic activity of equine antibacterial sera (antipneumococcic, antimeningococcic, etc.) is generally contained in the less soluble euglobulin fractions. Bacterial antibody globulin preparations also "lyophilize" well and, on the addition of water, eventually dissolve completely. The restored product shows little or no loss of potency. However, whether it is due to the particularly large molecular size of the antibody protein molecule or to the relatively

lower solubility of euglobulins, or to both factors, "lyophile" bacterial antibody globulin preparations require a longer time to dissolve than do any other class of "lyophile" serum or serum derivatives. They are, therefore, the least desirable for clinical use.

Some examples of the time required to dissolve representative classes of "lyophile" serum preparations are given in Table III. Restoration time depends on several factors: nature of the dry product (serum or globulin), protein concentration of the liquid preparation, thickness of shell or surface/volume ratio of frozen mass, and uniformity of drying (absence of fused spots). It should be noted that there is often a wide variation in the restoration time between the individual containers of the same lot of a given product. This is presumably due to variations in the thickness of the shell and occasionally to the presence of a fused spot which indicates drying of the serum in that spot from the liquid state. Such fused spots take longer to dissolve.

TABLE III. RESTORATION TIMES OF VARIOUS "LYOPHILE" PREPARATIONS

| DESCRIPTION OF PRODUCT | VOL. RESTORED TO C.C. | NUMBER OF CONTAINERS TESTED | RESTORATION TIME (MIN.) | REMARKS |
|---|---|---|---|---|
| Antimeningococcic serum, 30 c.c. | 15 | 10 | 3, 6, 13, 4, 4, 4, 4, 5, 17, 4 | Unconcentrated serum restored to one-half original volume |
| Antidysenteric serum, 50 c.c. | 50 | 4 | 4, 6, 3, 4 | Unconcentrated serum |
| Antidysenteric serum, 25 c.c. | 25 | 2 | 27, 30 | Serum concentrated two times prior to desiccation in market container |
| Diphtheria antitoxic globulin, 5 c.c. | 5 | 4 | 25, 30, 40, 32 | Purified and concentrated by enzymatic process; total solids, 17.7 per cent |
| Tetanus antitoxic globulin, 10 c.c. | 10 | 5 | 25, 20, 27, 32, 30 | Purified and concentrated by salting out; total solids, 17.5 per cent |
| Tetanus antitoxic globulin, 13.8 c.c. | 8 | 5 | 5, 5, 4, 5, 6 | Diluted to 6.0 per cent total solids prior to desiccation |
| Pneumococcic antibody globulin | 10 | 5 | All $> 60$ | Purified and concentrated by Felton's method; total solids, 7.7 per cent |

The results in Table III indicate that the most advantageous types of "lyophile" serum product in final containers for use by the clinicians appears to be whole serum. For certain antitoxic globulins, it has been found expedient to dilute the preparation to a protein content of from 5 to 6 per cent prior to desiccation. In most cases, such antitoxic globulins will dissolve within five minutes or thereabouts.

In our laboratories, a desirable limit of restoration time (average) of five minutes has been set arbitrarily for all "lyophile" products used clinically. Human plasma, guinea pig complement, and most antitoxic and antibacterial whole sera meet this standard without much difficulty.

## SUMMARY

1. Unprocessed (whole) sera taken as a class are admirably adapted to the "lyophile technique." The dehydrated products show no substantial loss

of potency as a result of the desiccation process; the products are restored rapidly and completely and the preservation of potency is maintained under adverse temperature conditions.

2. The removal of water from sera by the "lyophile technique" does not completely arrest or stop all subsequent chemical changes but certainly does retard them. The dry serum forms a system which eventually proceeds to a state of equilibrium which is determined by the temperature, pressure, moisture content, and other energy factors to which the serum is subjected.

3. The maximal residual moisture content in "lyophile" sera was arbitrarily set at 1.0 per cent, a figure now accepted and adopted by the National Institute of Health. This figure seems reasonable and allows a margin of safety for "lyophile" sera kept for long periods of time at a temperature higher than 5° C.

4. The potency of "lyophile" serum is retained for a longer period as compared with the same serum stored in the liquid state. This increased stability is least at from 2° to 5° C., more marked at room temperature and very marked at 37° C.

5. The most advantageous type of "lyophile" serum produced in final containers for use by the clinicians appears to be whole serum. Antibody globulins, unless diluted to a protein content of from 5 to 6 per cent prior to desiccation, are impractical for clinical use because of the excessively long time required to dissolve them.

6. The conversion of liquid sera to the "lyophile" form involves an expense which is reflected in the cost of the product. This added cost is justified for some products but not for others. The lability of the particular product and the temperature to which it is exposed are important determining factors.

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## PROGRESS

# FUNDAMENTAL INFORMATION ON THE MECHANISM OF SPECIFIC TUBERCULO-IMMUNITY

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SPECIFIC tuberculo-immunity in tuberculosis has been recognized for over half a century, and yet today its significance is discounted by many, apparently because we are still unable to measure its potency in man by any known safe method. This is not surprising, however, when we realize that certain groups of tuberculologists insist that tuberculology is so primitive that the value of any chemo- or biotherapeutic agent for tuberculosis in man must be proved by experimental test in the tuberculous patient and that no amount of animal experimentation can yield satisfactory conclusions. This would be to deny the similarities and differences noted for man and the lower forms of life as of value.

In a brief history of specific tuberculo-immunity,[1] tribute was paid to the pioneers in this field: Joseph Parrot, who announced the result of investigations concerning the relationship between pathologic changes in the lungs and in the tracheobronchial lymphatic glands; Robert Koch, who demonstrated for the first time the immune phenomenon in animals; Emil von Behring, whose daring Jennerization of cattle with a "bovo-vaccine" brought condemnation because the milk for infant immunization contained viable virulent human tubercle bacilli; Paul H. Roemer, whose monumental contributions led to the view of the unity of specific immunity and tuberculin hypersensitiveness, which later guided Allen K. Krause in the same view, that tissue allergy and immunity are the attributes of animals with tubercle; Anton Ghon, whose monograph stressed the significance of the primary tubercle and was the basis of the elaborate immunobiologic conceptions of Karl Ranke; Edward L. Trudeau; Gerald B. Webb; and Calmette. Aside from the vaccination endeavor, Calmette's greatest scientific achievement was to broach the avirulence of tubercle bacilli, although he did not settle it convincingly.

### TUBERCULIN

For simplicity and clarity in defining tuberculosis, a brief presentation regarding the practical significance of tuberculin is a pertinent introduction here because, in the past, tuberculin has been misleading in the clear understanding of the mechanism of tuberculosis.

Naturally tuberculin and its effects confused us a great deal, but in 1940[2] we noted that tuberculin was not appreciable in liquid mediums until after maximum growth of tubercle bacilli had occurred, and then it increased in the liquid to a maximum with aging of the bacillary mass in from six to ten months.

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At that time we suggested it might be a product of autolysis of the bacilli, which was definitely proved later with the preparation of autolytic tuberculin.[3]

In 1939[4] we pointed out that specific immunity against virulent infections could not be produced by pure tuberculoprotein, by highly concentrated filtrates (ultradialysis), or by alum-treated filtrates, and that these materials possess no appreciable primary toxicity for normal animals. However, a primary intravenous injection of tuberculin does not sensitize to a second intracutaneous injection with a fairly large test dose (0.1 mg.) of tuberculin. A reaction to tuberculin can be obtained in a tuberculous animal with as little as 0.000,005 mg. of tuberculin. A primary intravenous injection of tuberculin (2 mg.) sensitizes to a lethal intravenous provocative dose (anaphylactically) of as little as 1 mg. of tuberculin. Production of cutaneous hypersensitiveness to tuberculin requires a small amount of avirulent bacilli, a large amount of heat-killed bacilli (100 mg.), and very small amounts of virulent bacilli (the factors of multiplication and tuberculous involvement complicate the latter picture). In 1940[5] it was pointed out that tuberculin-sensitized animals do not develop an acute (anaphylactic) shock after the injection of washed young tubercle bacilli, while bacillary-sensitized and tuberculous (allergic) animals succumb to these injections. At the same time, it was shown that the tuberculin (anaphylactic) sensitization can be transferred passively from a high percentage of the donors, while the bacillary and tuberculous (allergic) principles are never transferable passively to normal recipients. Likewise, at that time tuberculin desensitization or immunization to the skin tuberculin reaction exerted no influence upon the specific tuberculosis immunity. The same year[6] it was shown that the phenomenon of local skin reactivity (Shwartzman) appeared to be of no significance in tuberculosis intoxication. It was also found that animals sensitized to tuberculin do not lose this anaphylactic sensitivity upon infection with virulent tubercle bacilli and the injection of avirulent tubercle bacilli. Thus, for the first time, the two reactions, anaphylactic and allergic hypersensitiveness, were demonstrated to coexist in the same animal. In 1941[7] it was noted that "although the specific toxicity in tuberculosis cannot be described definitely as yet, for lack of information regarding the part played by the products of tubercle bacilli liberated in vitro (tuberculin) but apparently not liberated in appreciable amounts in vivo, desensitization with these products (tuberculin) presents a fascinating problem, the exact nature and significance of which will have to be disclosed." The evidence questions whether the active constituent of in vitro natural filtrate from the growth of tubercle bacilli is liberated in vivo. In spite of this, desensitization of bacillary-tuberculo-allergically sensitized animals can be accomplished by appropriate treatment with tuberculin, so that animals thus prepared do not show a local specific skin reaction to tuberculin and are likewise protected against a lethal general allergic shock intoxication with tuberculin. The protective materials are contained in the blood of desensitized animals, and this protection can be transferred to a tuberculo-allergic-sensitive animal with the blood from a desensitized tuberculo-allergic animal. Tuberculo desensitization with primarily nontoxic amounts of tuberculin exerts no beneficial or detrimental effect upon tuberculosis or upon specific tuberculo-

immunity. By 1942[8] we began to see definitely the fallacy of tuberculin therapy for treating tuberculosis and also began to understand more fully the desensitization or immunization with tuberculin against tuberculin allergic intoxication as an extraneous phenomenon to tuberculosis and of no practical import. One year later[9] it was noted that tuberculin therapy in man is not warranted by any evidence available at present from animal experiments. Then it was recognized that while we could desensitize against tuberculin-allergic intoxication with tuberculin, this exerted no appreciable effect upon the resulting tuberculosis, and the reaction to viable tubercle bacilli was not retarded. We were able to define two separate types of tuberculo-allergic hypersensitiveness: (1) that elicited by tuberculin, which seems to be of no practical importance in tuberculosis as it occurs naturally, and (2) that elicited by viable tubercle bacilli and which naturally assumes importance in the disease. These experiments clarify the reasoning of investigators in the past who assumed that tuberculo-allergy and tuberculo-immunity were synonymous or that they represented closely related phenomena. The deductions were not correct, however, since they were drawn from the use of tuberculin as the test agent for allergy. To complete the tuberculin picture, in 1943[10] it was shown further that passive transfer of blood of tuberculin-immune guinea pigs to normal animals prevented tuberculin sensitization to tuberculin anaphylactic shock, and that passive transfer of blood of immune or anaphylactic-hypersensitive guinea pigs with or without the addition of tuberculin failed to protect tuberculin-hypersensitive guinea pigs from the effect of the lethal tuberculin anaphylactic shock.

## SPECIFIC TUBERCULO-IMMUNITY

Our close association with specific tuberculo-immunity began in about 1935.[11] Without fully understanding avirulent tubercle bacilli, we were able to satisfy ourselves of the significance and potency of specific tuberculo-immunity by 1936.[12] Then it was shown for the first time that very small numbers of virulent tubercle bacilli mixed with large amounts of fine suspensions of avirulent tubercle bacilli would produce tuberculosis unhampered in the guinea pig, that tubercle was a function of dead bacillary bodies, and that viability played no decisive part in this except in so far as bacillary bodies were produced. We noted also that resolution occurred with tubercles formed by avirulent bacilli, and that previous injections of avirulent human and bovine tubercle bacilli retarded the subsequent infections with virulent human or bovine tubercle bacilli, while heat-killed avirulent and virulent bacilli exerted no such effect. Avirulent human and bovine tubercle bacilli were proved to produce no progressive lesions in man, and the bacilli lost their viability in these lesions in about six months. The reactions to viable avirulent tubercle bacilli in man showed a sequence of changed reaction upon repeated injections at definite intervals similar to the changes noted in animals and occurring coincidentally with the development of artificial immunity in these animals.[13] It was pointed out that there is an apparent paradox resulting from this reaction in that the lesions produced by small and large numbers of virulent tubercle bacilli are retarded by the immune reaction as a result of preventing or retarding the multiplication of

virulent bacilli—*less lesion to virulent infection;* and, on the other hand, there is a greater tissue reaction in the immune organism as compared with the nonimmune to large numbers of avirulent bacilli incapable of multiplying in the tissues—*more tissue reaction to avirulent bacilli.* Thus, conclusions drawn from pathologic findings alone, without knowing the quantity of either one of these two decisive factors concerned and necessary for interpretation (the number of bacilli producing the reaction or the existing immune condition present), can be of no practical value. In 1937[14] we became more fully aware that these two reactions were separate entities, one which is the expression of a specific tuberculo-immunity and the other, still vague, which we erroneously referred to as "a type of protein intoxication" that does not become evident until large amounts of tubercle bacilli are either injected or disseminated internally after a primary contact with viable tubercle bacilli. We still referred to this as anaphylactic shock or reactions at that time. By 1938[15] we became more confident of the lack of multiplication of avirulent tubercle bacilli and talked about the toxicity of these bacilli and the absence of infectivity with the possibility of resolution following the intravenous injection of excessive amounts into normal nonimmune guinea pigs. This led to our definition of an avirulent tubercle bacillus on the basis of the lack of ability to multiply in a highly susceptible host when injected intravenously. The property of avirulence and virulence of mammalian tubercle bacilli was consistently stable within a reasonable time, many months at least, on artificial mediums or in the animal body when properly tested.[16] Changes in virulence of mammalian tubercle bacilli appear to be a gradual transition and usually occur only slowly over many generations of progency of a pure strain. No guaranteed means of a ready or permanent transition appears to be available as yet. By 1939,[4] we stated that "There is a striking quantitative difference between the specific immune and the concomitant allergic or anaphylactic features of tuberculosis which will eventually have to be given independent consideration in the complete evaluation of tuberculosis in man, since one is protective and the other shows a peculiar type of still unsolved intoxication." The following year[17] we said that the bacillary body sensitizes primarily to tuberculo-allergy, while the natural filtrate containing tuberculoprotein sensitizes to anaphylaxis and provokes anaphylactic shock and allergic intoxication but does not sensitize to allergy nor specifically immunize against virulent infection. At that time we concluded that tuberculo-anaphylaxis, tuberculo-allergy, and tuberculo-immunity were separate and apparently unrelated biologic phenomena. We were now able to demonstrate[5] that the skin (allergic tuberculin) reaction may be entirely absent in animals which still retain their specific tuberculosis immunity, and vice versa, and that desensitization with tuberculin to the skin tuberculin reaction exerts no influence upon the specific tuberculosis immunity. We were also well aware at this time that the specific immunity persisted unabated in its effects for well over two years.[18] In 1943[19] it was noted that "In no disease are the relative amounts of interacting factors of more decisive significance than they are in tuberculosis; and yet no exact scientific measure of the interacting relations of causative agent and host exists which can be applied readily in evaluating these factors in man." As a result,

there are those who, even to the present time, believe in an increased susceptibility following a primary infection, and this, a priori conclusion, is based on the fact that in some cases the disease progresses to an advanced stage. They fail to recognize the factors so involved and especially the possibilities in the fate and circumstances of heavily infected foci. No immunity is or can be absolute. It has its quantitative limitations, as it does in tuberculosis also. However, specific tuberculosis immunity shows definite quantitative aspects as gauged by graded virulent infecting injections and graded specific immunizing vaccinations with viable avirulent tubercle bacilli. In concluding the consideration of specific tuberculo-immunity, it might be added further that, unlike the tuberculin phenomenon, it has not been possible in our experiences to transfer passively specific tuberculo-immunity perceptibly to normal recipients.[20] Likewise, the specific immunity is not passed in utero from mother to offspring.[21] Thus, immunization must be active to be appreciably effective for the recipient.

## SPECIFIC TUBERCULO-ALLERGY

In tuberculosis, we are dealing with a disease of conspicuously dual aspects, specific tuberculo-immunity and a specific tuberculo-allergy, aside from the usual bacillary and pathologic anatomic incriminations. We could not dwell long upon vital interpretations in tuberculosis without being confronted by the phenomenon of tuberculo-allergy. We were aware that intoxication in full-blown disease, as in many destructive diseases, may be associated with host tissue destruction and the absorption of disintegration products, but we also knew that normal animals could tolerate inconceivably large amounts of viable avirulent tubercle bacilli intravenously without violent response. Tuberculin apparently was not liberated in the host body and, even if it had been, desensitization or immunization to tuberculin was possible and did occur according to reliable scientific experimental test. We were aided by the preparation of purer tuberculins and bacillary suspensions in the solution of this problem. Our conceptions first began to take shape in 1940[17] with the recognition that tuberculo-anaphylaxis, tuberculo-allergy, and tuberculo-immunity showed distinctive characteristics, pointing to their being separate and apparently unrelated phenomena. At that time it was noted that bacillary-sensitized or tuberculous animals do not respond to an injection of tuberculin after an appropriate incubation period by an acute anaphylactic shock reaction but do reveal a protracted (allergic) intoxication to a suitable amount of tuberculin with fatal issue after from one to three days except when the tuberculous involvement is very marked, when it occurs more rapidly. Also, tuberculin-sensitized animals do not develop an acute (anaphylactic) shock after the injection of young washed tubercle bacilli, while bacillary-sensitized and tuberculous (allergic) animals succumb to these injections. The bacillary and tuberculous (allergic) principals are never passively transferable to normal recipients. The lethal effect (allergic) of tuberculin in tuberculous animals appears to depend upon the time and amount of involvement following infection with virulent tubercle bacilli. Peculiarly enough, while it is possible to desensitize to tuberculin-allergic intoxication by means of tuberculin and also thus abolish

the local skin reaction (tuberculin reaction), this does not alter the specific immunologic condition appreciably nor does it prevent bacillary intoxication.[5]

In 1942[22] a rather interesting set of experiments was performed in which it was shown that the addition of certain paraffins markedly accentuated the property of inducing allergic hypersensitiveness to tuberculin by heat-killed tubercle bacilli, verifying earlier work by Saenz, but these paraffins exerted little effect in accentuating the specific tuberculo-immunity produced by tubercle bacilli either dead or alive. In 1943[9] we became more fully aware of two types of tuberculo-allergic hypersensitiveness: (1) that elicited by tuberculin, seemingly of no practical import, and (2) that elicited by viable tubercle bacilli, important naturally in the disease. It was noted also at this time that preliminary attempts to desensitize or immunize against the bacillary allergic hypersensitiveness by using dead or viable tubercle bacilli failed. In 1943[21] it was noted that the specific tuberculo-allergic hypersensitiveness to tuberculin was not passed from mother to offspring in utero nor could it be transferred passively by intravenous injection of blood.[20] Before passing on to the implication of specific tuberculo-bacillary allergic hypersensitiveness in man, I will summarize our final attempt (in 1944[23]) to desensitize against this condition in animals. Repeated intravenous injections of viable avirulent tubercle bacilli resulted in no appreciable reduction of hypersensitiveness either to tuberculin or to viable bacillary bodies but rather in an accentuation of the allergic hypersensitiveness; likewise, an attempt to desensitize tuberculo-bacillary-hypersensitive animals by means of repeated subcutaneous injections of heat-killed tubercle bacilli in mineral oil resulted in only a slight accentuation of the allergic hypersensitiveness. Dead (heat-killed) tubercle bacilli suspended in saline and subcutaneously injected repeatedly showed no appreciable effect on the tuberculo-bacillary allergic hypersensitiveness; however, when injected intravenously, a depression of the cutaneous allergic hypersensitiveness to tuberculin and bacillary bodies occurred (believed due to effect on general vascular or inflammatory response of animals). Dead avirulent tubercle bacilli repeatedly injected intravenously revealed no appreciable effect on the development of the tuberculous disease resulting from subcutaneous infection with highly virulent tubercle bacilli, but these same injections appeared to exert a slight detrimental effect on the specific immunity resulting from vaccination with viable avirulent tubercle bacilli which may be accounted for by the ability of the dead bacillary bodies to produce tubercles and productive tissue reactions.

Since 1918, it has been our custom to study carefully tubercle bacilli freshly isolated from human sources to determine their virulence. In 1942[24] we had an unusual opportunity to observe a classical example of death due to tuberculo-allergic bacillary hypersensitiveness in a patient who, at the time of death, harbored only avirulent tubercle bacilli. The spread of these bacilli resulted in the fatal tuberculo-allergic bacillary intoxication, a protracted death characterized by the same symptoms noted for such death in experimental animals. Post-mortem examination established the anatomic diagnosis of fibrocavernous tuberculosis in both lungs. The heart showed no abnormalities beyond a slight right hypertrophy. The significant pathologic changes were a pronounced marginal acute hyperemia

in the left lung not, however, attaining a pneumonic stage. This was the early result of a bacillary allergic reaction, the intoxication resulting in the collapse and death in a patient harboring only avirulent tubercle bacilli in the lungs in sufficient amount to cause the terminal lethal allergic intoxication. This patient with avirulent tubercle bacilli had what appears to be a clear-cut case of final lethal tuberculo-allergic bacillary intoxication in the human being, a condition which may arise in any active open case of tuberculosis. As the disease advances, the bacilli become available in sufficient amount for a sudden massive mobilization toward actively absorbing or reacting sites. To counteract this, we have as yet found no immediate treatment, although all abolition or collapse measures used at present serve as preventives against these accidents of tuberculosis.

## SUMMARY

1. We have verified and demonstrated the following:

(a) A tuberculin anaphylactic hypersensitiveness exists and is produced by tuberculoproteins (tuberculin) appropriately injected. It does not appear to be of any significance in the natural picture of tuberculosis in man or animals and is mainly of academic interest.

(b) Tuberculin (tuberculoprotein) anaphylactic hypersensitiveness can be transferred passively with the blood and is passed from mother to offspring in utero.

(c) Tuberculo-anaphylactic shock and tuberculo-allergic shock produced by the injection of tuberculin (tuberculoprotein) present entirely different clinical and pathologic manifestations and can be demonstrated in the same animal under appropriate conditions.

2. We believe that we have verified and demonstrated the existence of a relative specific tuberculo-immunity produced by (a) viable (avirulent human and bovine) tubercle bacilli, but not by (b) heat-killed nonviable tubercle bacilli by (c) the filtrate from growing tubercle bacilli on a nonprotein synthetic medium or by (d) tuberculoproteins (tuberculins).

This specific tuberculo-immunity must be produced actively and persists over four years; it cannot be transferred passively with the blood; and it does not pass from mother to offspring in utero.

There is no means of measuring the specific immunity at present except by virulent infection.

3. We have verified and demonstrated the existence of a specific tuberculo-allergy which is produced by (a) viable tubercle bacilli and by (b) heat-killed nonviable tubercle bacilli which are more efficient when suspended in paraffin or mineral oils.

This specific tuberculo-allergy appears to be no measure of immunity, although it may be produced during infection coincident with it. When produced by avirulent tubercle bacilli, tuberculo-allergy reaches a maximum at about two months and may disappear in about six to twelve months. It cannot be transferred passively with the blood nor is it passed from mother to offspring in utero.

Desensitization or immunization against allergic tuberculin hypersensitiveness can be achieved by appropriate injections of tuberculin.

Allergic bacillary hypersensitiveness is not affected by tuberculin injections, so that tuberculin therapy appears futile for this purpose practically.

Specific tuberculo-immunity and specific tuberculo-allergy play a significant though individual part in the picture of clinical tuberculosis.

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# LABORATORY METHODS

## A RAPID TEST FOR DISTINGUISHING HUMAN FROM COW'S MILK BASED UPON A DIFFERENCE IN THEIR XANTHINE OXIDASE CONTENT

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HUMAN milk is purchased and distributed for infant feeding by many institutions throughout this country. The price paid to mothers for their milk is about fifteen times the cost of an equal amount of cow's milk. Thus, the dilution of human milk with cow's milk becomes a profitable procedure, and one which must be guarded against by the purchaser. We present here a simple test for detecting the admixture of human and cow's milk. It is based upon the fact that human milk is practically devoid of the enzyme xanthine oxidase, while cow's milk, even after commercial pasteurization, is rich in this enzyme. The xanthine oxidase activity of a milk sample can, therefore, be used as an index to its origin.

### EXPERIMENTAL

A comparison of the xanthine oxidase activity of human and cow's milk was first made using a manometric procedure. The oxygen consumption of samples in the presence of hypoxanthine as a substrate was followed in the manner employed by Ball.[1] The test was arranged as follows: 2 c.c. of milk and 1 c.c. of 0.1 M phosphate buffer, pH 7.2, were placed in the main chamber of a conical Warburg manometer flask. The side arm of this flask contained 0.2 c.c. of a 0.05 M solution of hypoxanthine dissolved in 0.05 M NaOH. After temperature equilibration of the flasks in the water bath which was maintained at 37° C., readings were taken for a five-minute period in order to be certain that no oxygen consumption occurred in the absence of the substrate. The substrate was then added from the side arm, and readings were made every ten minutes for a total period of one-half hour.

The results of the analyses of twenty-six different samples of human milk and six different samples of cow's milk are shown in Table I. The samples of human milk were all unpasteurized and obtained fresh from the Mothers' Milk Directory at the Boston Lying-In Hospital.* The samples of cow's milk were purchased on the open market in Boston and represent milk from six different dairies. All samples were stated to be pasteurized.

The results clearly indicate that there is a marked difference in the xanthine oxidase activity of cow's and human milk. Only three of the twenty-six samples of human milk showed any measurable oxygen consumption, and in these the

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*Courtesy of Dr. F. J. Stare.

TABLE I. COMPARISON OF THE XANTHINE OXIDASE ACTIVITY OF HUMAN AND COW'S MILK

| SAMPLE | COW'S MILK | HUMAN MILK |
|---|---|---|
| 1 | 64 | 3 |
| 2 | 128 | 4 |
| 3 | 53 | 5 |
| 4 | 62 | 0* |
| 5 | 84 | 0* |
| 6 | 90 | 0 |
| 7 to 26 | | 0 |

Results are expressed as cubic millimeters of $O_2$ consumed in thirty minutes at 37° C. and pH 7.2 by 2 c.c. of milk with hypoxanthine as substrate.

*Colostrum.

amount of oxygen consumed was insignificant in comparison to that observed for cow's milk. For all practical purposes, it may be said that human milk is devoid of xanthine oxidase. The variation in activity shown by the different samples of cow's milk may be, in part, due to differences in pasteurization techniques at the various dairies. However, it has been reported[2] that the xanthine oxidase activity of an individual cow's milk is quite variable from day to day.

The xanthine oxidase activity of a milk sample may thus be used as an index to its source. If a sample of milk reputed to be human shows an appreciable xanthine oxidase activity, it is extremely likely that some cow's milk is present. The manometric procedure outlined may be employed for the purpose of assaying the xanthine oxidase activity of milk samples. A simpler method, however, is one based on the reduction of methylene blue.

The apparatus necessary to measure xanthine oxidase activity by the methylene blue method consists of ordinary pipettes, Thunberg tubes, and a water suction pump. The solutions necessary are (a) 0.005 per cent methylene blue solution and (b) 0.05 M xanthine or hypoxanthine in 0.05 M sodium hydroxide. The test is just about twice as rapid if hypoxanthine is available* for use.

To perform the test, 2 c.c. of milk are placed in the Thunberg tube† and 0.5 c.c. of the methylene blue solution is added. In the side arm or hollow stopper of the Thunberg tube is placed 0.3 c.c. of the xanthine solution or 0.2 c.c. of

TABLE II. REDUCTION OF METHYLENE BLUE BY MIXTURES OF HUMAN AND COW'S MILK WITH XANTHINE AS SUBSTRATE

| PERCENTAGE COW'S MILK IN MIXTURE | METHYLENE BLUE DECOLORIZATION TIME (MIN.) | |
|---|---|---|
| | 18° C. | 23° C. |
| 100 | 14 | 10 |
| 50 | 15 | 11 |
| 33 | 17 | 12 |
| 25 | 21 | 15 |
| 20 | 22 | 16 |
| 16 | 25 | 18 |
| 14 | 28 | 20 |
| 0 | ∞ | ∞ |

*Hypoxanthine is not available on the market at present, but xanthine may be purchased from Eastman Kodak Co., Rochester, N. Y.

†If Thunberg tubes are not available, a fairly satisfactory test may be run by employing ordinary test tubes containing from 2 to 3 c.c. of mineral oil. All solutions are pipetted into the tube under the oil and carefully mixed. The layer of oil serves to prevent access of air which would reoxidize the leucomethylene blue as it is formed.

hypoxanthine. The tube is now evacuated at the water pump, the temperature of the tube adjusted if appreciable cooling has resulted, and the substrate is mixed with the milk by inverting the tube. The time for conversion of the methylene blue to its leuco form is noted. A blank should be run with each sample in order to rule out any reduction due to bacterial action. Except for the addition of substrate, the blank is treated in the same fashion.

The most favorable temperature for the action of xanthine oxidase is 40° C. The reaction takes place, however, perfectly well at room temperature so that a water bath need not be employed unless more rapid results are desired. In Table II data are given showing the methylene blue reduction times at two different temperatures of a series of mixtures of human and cow's milk with xanthine as substrate. The cow's milk used to make these mixtures was the sample that showed the lowest xanthine oxidase activity by the manometric test (Sample 3, Table I).

It is obvious from these results that it is possible to detect readily human milk which has been diluted with one-seventh its volume of cow's milk. We have readily detected mixtures containing 9 per cent cow's milk which were prepared for us as a test of the method by the Mothers' Milk Directory. Lower dilutions than these could probably be detected, but it is doubtful that they would ever been encountered where willful dilution of human milk is occurring.

The test outlined here is, of course, dependent upon pasteurized cow's milk showing xanthine oxidase activity. It is imperative, therefore, that this point be checked for each geographic region in which the test is to be employed. In general, the more extensive the pasteurization period and the higher the temperature employed, the lower will be the xanthine oxidase activity of the milk. Cow's milk which has been boiled will show no xanthine oxidase activity and the test will, therefore, fail to detect dilution when this procedure has been employed.

## SUMMARY

1. Human milk samples from twenty-six individuals showed little or no xanthine oxidase activity. Pasteurized cow's milk, by comparison, is rich in this enzyme.

2. A simple test based upon xanthine oxidase activity is described which permits purchasers of human milk to detect whether dilution of the sample with cow's milk has been performed. The test will consistently detect as little as 10 per cent admixture of cow's milk with human.

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# MODIFICATION OF THE GUTMAN AND GUTMAN METHOD OF ESTIMATING "ACID" PHOSPHATASE ACTIVITY

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THE determination of acid phosphatase in the blood is now generally recognized as an important test for the diagnosis of carcinoma of the prostate gland. According to our experience, however, the possibility of serious error is not sufficiently eliminated in the procedures used at present in clinical laboratories. In this paper we shall describe an improved technique in which the sources of error inherent in the current procedures have been eliminated.

The main difficulty arises from the instability of the phenyl phosphate which represents a suitable substrate for the determination of acid phosphatase. We found that considerable amounts of phenol are formed when a solution of phosphate is kept in the refrigerator for several weeks at the optimum pH of acid phosphatase. Even during the incubation period of one hour at 37° C. hydrolysis is significant. We have been unable to replace phenyl phosphate by more stable phosphoric esters. Glycerophosphate, which is very stable, was found to give unsatisfactory results in the quantitative determination of acid phosphatase in blood. According to Gutman and Gutman,[1] and according to our own investigations, it is hydrolyzed at a much slower rate than is phenyl phosphate. In addition, the hydrolysis of glycerophosphate can be studied only by means of phosphate determination which is much less sensitive than phenol determination. Even in very active cases of prostatic carcinoma, the values of acid phosphatase activity are not very high measuring phosphate split from glycerophosphate. Consequently, the small increases of inorganic phosphate caused by the incubation of glycerophosphate with serum at the optimum pH of acid phosphatase cannot be determined with sufficient accuracy.

For these reasons it appeared to be advisable to continue the use of phenyl phosphate as a substrate and to improve the current procedure with the view of keeping the spontaneous hydrolysis of phenyl phosphate at a minimum and to account for the unavoidable formation of phenol by suitable control tests. The original directions of Gutman and Gutman[2] were therefore modified in two ways: (1) The use of a substrate buffer mixture as a stock solution was abandoned. It was found that the spontaneous hydrolysis during the storage could be sufficiently reduced if a solution of disodium phenyl phosphate in water and the buffer solution were kept separately in the refrigerator and mixed only immediately before the phosphatase determination. (2) A control test containing buffer substrate mixture and water was incubated concurrently with the sample which contained phosphatase. The amount of phenol formed in the serum blank and in the substrate control was deducted from that formed in the presence of phosphatase.

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### REAGENTS

1. Citrate buffer, pH 4.9. Dissolve 18.9 Gm. of citric acid in 500 c.c. of water and add 180 c.c. of N. NaOH and 100 c.c. of N/10 HCl. Make up to 1 liter. (pH of buffer should be checked.)
2. Substrate, 1 per cent disodium phenyl phosphate (keeps about two weeks in icebox).
3. Dilute phenol reagent (Folin & Ciocalteau). Dilute 1 volume with 2 volumes of water.
4. 20 per cent sodium carbonate.

### METHOD WITHOUT SUBSTRATE CONTROL

| BLANK | TEST |
|---|---|
| 9 c.c. buffer | 9 c.c. buffer |
| 1 c.c. substrate | 1 c.c. substrate |
| .5 c.c. serum | .5 c.c. serum |
| Immediately add 4.5 c.c. phenol reagent | Incubate one hour at 37° C. |
| Filter | Add 4.5 c.c. phenol reagent |
| | Filter |

| BLANK | TEST | STANDARD |
|---|---|---|
| 10 c.c. filtrate | 10 c.c. filtrate | 10 c.c. standard |
| 2.5 c.c. 20 per cent $Na_2CO_3$ | 2.5 c.c. 20 per cent $Na_2CO_3$ | 2.5 c.c. 20 per cent $Na_2CO_3$ |

Incubate all at 37° C. for five minutes and read immediately

The standard solution may or may not be necessary, depending on whether a calibrated photometer is available. The 10 c.c. standard solution should contain .04 mg. of phenol, 3 c.c. of phenol reagent, and water up to 10 c.c.

The milligrams per cent of phenol found in the blank subtracted from the milligrams per cent of phenol found in the test give the milligrams per cent of phenol presumably split by the enzyme and are considered "King units"[3] of activity.

We employed this method for some time and obtained what appeared to be clinically reliable results. However, when a new supply of disodium phenyl phosphate was used, we suddenly began to get high results almost routinely. Further investigation of this difficulty led to a still more improved procedure which we are at present employing.

We discovered that upon incubation of substrate alone (disodium phenyl phosphate*) definite values of phenol were hydrolyzed.† This may be explained by the action of an acid medium or by the presence of impurities in the substrate. In any case, such a value must necessarily be deducted from the test regardless of what causes this hydrolysis. This necessitated a second blank made up of substrate and buffer in addition to the original blank. The latter two results are added together and subtracted from the test.

*Obtained from Paul-Lewis Laboratories, Inc., Milwaukee, Wis.

†Gutman and Gutman[1] recommend the use of a highly purified phenyl phosphate. Such a reagent is not commercially available. In view of the great clinical importance of the test, it is desirable to have a method which can be accurately performed even in smaller hospitals which do not have the facilities for checking or improving the purity of commercial phenyl phosphate. The technique presented in this paper was devised for this purpose and appears to be the most practical way of overcoming the above difficulties.

IMPROVED PROCEDURE

| $B_1$ (SERUM BLANK) | $B_2$ (SUBSTRATE BLANK) | TEST |
|---|---|---|
| -- | 9 c.c. buffer | 9 c.c. buffer |
| 10 c.c. water | .5 c.c. water | -- |
| -- | 1 c.c. substrate | 1 c.c. substrate |
| .5 c.c. serum | -- | .5 c.c. serum |
| Immediately add 4.5 c.c. phenol reagent | Incubate one hour at 37° C. | Incubate one hour at 37° C. |
| Filter | Add 4.5 c.c. phenol reagent | Add 4.5 c.c. phenol reagent |
| | Filter | Filter |

Take 10 c.c. of filtrate from $B_1$, $B_2$, Test, and Standard if necessary (make up as previously described). The color is developed by the addition of sodium carbonate and incubation as described in the former method. The milligrams per cent found in $B_1$ plus $B_2$ subtracted from the milligrams per cent of phenol found in Test give the milligrams per cent of phenol split by acid phosphatase, which become "King units" by definition. We have had very good results employing a Cenco photelometer which we calibrated for phenol.

## DISCUSSION

The results in Table I show that larger values are obtained by the method without substrate control. This is of special interest because most of the values are above the original limit of normal values published, 1 to 4 King units. By the improved procedure, much smaller figures are realized. The values do not seem to run above 4 except when pathology is found, as shown by Table II. Only two patients of eleven revealed no immediate evidence of carcinoma of the prostate.

TABLE I

| CASE | VALUES OBTAINED WITHOUT SUBSTRATE CONTROL (KING UNITS) | IMPROVED PROCEDURE (KING UNITS) |
|---|---|---|
| 1 | 6.6 | 2.1 |
| 2 | 3.5 | 0.15 |
| 3 | 0.9 | 0.3 |
| 4 | 3.9 | 0.9 |
| 5 | 4.8 | 2.7 |
| 6 | 4.2 | 0.9 |
| 7 | 4.8 | 0.3 |
| 8 | 3.6 | 0.3 |
| 9 | 4.2 | 0.9 |
| 10 | 6.3 | 3.3 |
| 11 | 10.8 | 2.4 |
| 12 | 8.4 | 0.6 |
| 13 | 8.7 | 0.6 |
| 14 | 9.9 | 1.2 |
| 15 | 9.0 | 0.0 |
| 16 | 8.4 | 0.3 |
| 17 | 10.2 | 2.1 |
| 18 | 10.8 | 0.6 |
| 19 | 12.6 | 3.3 |
| 20 | 3.9 | 0.6 |
| 21 | 6.6 | 0.6 |
| 22 | 13.8 | 3.3 |

TABLE II

| CASE | RESULT BY IMPROVED PROCEDURE | DIAGNOSIS OF CARCINOMA OF PROSTATE | METHOD |
|---|---|---|---|
| 23 | 5.7 | Positive | Surgery |
| 24 | 5.0 | Positive | Cystoscopy |
| 25 | 14.0 | Positive | Cystoscopy |
| 26 | 21.2 | Positive | Surgery |
| 27 | 5.3 | Positive | Surgery |
| 28 | 32.0 | Positive | Surgery |
| 29 | 6.3 | Negative | Surgery |
| 30 | 6.9 | Positive | Cystoscopy |
| 31 | 6.4 | Positive | Surgery |
| 32 | 4.7 | Positive | Surgery |
| 33 | 5.0 | Negative | Cystoscopy |

SUMMARY

The original method of Gutman for acid phosphatase has been altered in certain details. This was accomplished by using the buffer substrate as two solutions rather than as a mixture and by setting up an additional substrate buffer control. A comparison of values is given with and without this control. It is pointed out that false high values are realized by the original Gutman method simply because the substrate may be hydrolyzed by the acid medium itself even in the absence of acid phosphatase. The results of this improved procedure have been proved to be more reliable than other methods.

We wish to express our appreciation to Dr. Gerhardt Schmidt for valuable technical advice.

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# DETECTION OF TUBERCLE BACILLI IN SPUTUM; APPLICATION OF THE NAOH-ALUM METHOD, THE CLOROX METHOD, AND THE DIRECT SMEAR

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THE purpose of this study, as stated in an earlier publication,[1] was to compare the effciency of the NaOH digestion method, in which potassium alum is used as an aid in concentrating tubercle bacilli in sputum, with the efficiency of the clorox method and the direct smear. Identical specimens of sputum were studied by each of the three methods and the observations compared.

Comparisons of the NaOH, clorox, autoclave, and direct smear methods have been reported.[1, 2] The use of alum for chemical flocculation in the NaOH method, as described by Hanks and co-workers,[3] was not included in the previous studies. The autoclave method was not included in the present work.

## EXPERIMENTAL PROCEDURE

All preparations were made by one person to eliminate the personal factor as a variant. The specimens were allowed to settle, and some of the concentrated material was drawn up from the bottom in pipettes for preparing the direct smears. These smears were made as thick as was practical for finding and counting the organisms in the stained preparations. Measured and equal amounts of each specimen were used for the NaOH-alum and clorox methods. Two cubic centimeters of sputum were measured for each method.

The normal NaOH solution used for digestion had 0.2 per cent potassium alum added, as recommended by Hanks,[3] to aid in concentration of the acid-fast organisms. The flocculation provided a nice background in the stained preparation and seemed to aid in fixing the specimen to the slide. These advantages were obtained for the clorox method by moistening the applicator in clear blood serum before taking up the specimen material to prepare the smear.

In the staining procedure, the slides were immersed in a carbolfuchsin vat, held at 37° C., and left in this stain for thirty minutes. This was used, rather than steaming over a Bunsen flame for five minutes, to alleviate the handling of a large number of slides. A staining vat was also used for acid-alcohol and for the counterstain. Slides were washed under running water after each step in staining.

The three slides prepared for any specimen were examined consecutively. Thus, if there semed to be any gross discrepancies, a recheck could be made at once. At least 100 fields were carefully examined on each negative side. As a result, the total fields examined for each negative specimen were at least 300. This corresponds to the routine procedure of the Division of Laboratories, of the Tennessee Department of Public Health, in which approximately 300 micro-

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scopic fields are examined before a negative report is given for a sputum specimen.

Careful counts of the organisms per microscopic field were made in most instances. This was not done when the count per field was over 100. For counts between 100 and 300, the figure recorded was a careful estimate. Three hundred represented the maximum count recorded for any field. When the count per microscopic field was from one to fifty organisms, from five to twenty-five fields were counted and the average per field determined. The primary objective was a fair comparison between the methods employed.

Findings were recorded as positive when at least three typical acid-fast organisms were found by one or more methods. If less than three were found, another specimen was requested. The goal in this study was set at 100 positive specimens in order that simple percentage determinations could easily be made in comparing the efficiency of the various methods.

## OBSERVATIONS

Of 397 specimens examined, 283 were found negative by all methods, 100 were positive by one or more methods, and fourteen yielded less than three typical acid-fast organisms by the three methods combined. For each of the latter specimens, another specimen was requested.

The specimens found positive are of particular interest for purposes of comparison. Most specimens were sent to the laboratory for either diagnosis or a check on treatment. Eighty-four per cent of the specimens found positive were examined to aid in diagnosis and 12 per cent to check on treatment; for 4 per cent the purpose was not designated.

Seventy-seven of the 100 positive specimens were found positive by all of the methods used. The other twenty-three were found positive by one or two methods. These observations are given in Table I.

TABLE I. NUMBER OF SPECIMENS FOUND POSITIVE FOR TUBERCLE BACILLI BY METHODS INDICATED

| METHOD | NUMBER | PER CENT |
|---|---|---|
| All methods used | 77 | 77 |
| Clorox and NaOH-alum only | 12 | 12 |
| Clorox only | 6 | 6 |
| Clorox and direct only | 3 | 3 |
| NaOH-alum and direct only | 2 | 2 |
| NaOH-alum only | 0 | 0 |
| Direct only | 0 | 0 |
| Total | 100 | 100 |

From this table it may be seen that acid-fast organisms were found in 98 per cent of the 100 positive specimens by the clorox method, 91 per cent by the NaOH-alum method, and 82 per cent by the direct smear.

In Table II, it may be seen that the average count of organisms per microscopic field by the clorox method was unexceeded for 81 per cent of the specimens. The NaOH-alum method yielded an unexceeded count for 24 per cent of the specimens and the direct for 10 per cent.

A far more striking comparison for the three methods is observed in the

TABLE II. FREQUENCY OF HIGHEST COUNT OF TUBERCLE BACILLI PER MICROSCOPIC FIELD FOR POSITIVE SPECIMENS

| METHOD | PER CENT |
|---|---|
| Clorox | 68 |
| NaOH-alum | 13 |
| Direct | 6 |
| Clorox and NaOH-alum | 9 |
| Clorox and direct | 2 |
| Clorox, NaOH-alum, direct | 2 |
| Total | 100 |

average number of acid-fast organisms found per 100 microscopic fields. This comparison is based upon the seventy-seven specimens which were found positive by each and all of the three methods used.

The large number of fields counted by the direct method is due to the fact that it was necessary to examine more fields to find acid-fast organisms by this method.

## DISCUSSION

The results obtained show the same trend as have been shown in similar previous studies.[1, 2] For 188 specimens found positive in the combined studies made in this laboratory by the clorox method and by the NaOH or NaOH-alum method, the average number of organisms per 100 microscopic fields was 1,274 for the clorox method and 438 for the NaOH methods. The average count of 1,052 recorded in Table III for the clorox method as compared with 465 for the NaOH-alum method per 100 microscopic fields compares favorably with the over-all picture found in these studies.

TABLE III. AVERAGE NUMBER OF ACID-FAST ORGANISMS PER 100 MICROSCOPIC FIELDS FOR SEVENTY-SEVEN SPECIMENS POSITIVE BY ALL METHODS

| METHOD | FIELDS COUNTED TO FIND SPECIMENS POSITIVE | TOTAL ORGANISMS FOR FIELDS COUNTED | AVERAGE ORGANISMS PER 100 FIELDS |
|---|---|---|---|
| Clorox | 855 | 8,996 | 1,052 |
| NaOH-alum | 967 | 4,492 | 465 |
| Direct | 1,406 | 3,144 | 224 |

The fact that an average of 1,052 organisms per 100 fields was found in this study by the clorox method as compared with 1,274 by the same method for the combined studies seems to indicate that fewer organisms were present. In that case the increase in the average, 465 for the NaOH-alum method as compared with 438 for the combined studies by the NaOH methods, becomes somewhat more significant. The comparative increase in efficiency of the NaOH digestion method in this study is probably due to the use of alum flocculation for concentration of the tubercle bacillus. This is in accord with the report of Hanks[3] on the use of chemical flocculants as an aid in concentrating acid-fast organisms in laboratory specimens. However, the results still definitely favor the clorox method.

Acid-fast organisms were found in twelve of the 100 positive specimens by the NaOH-alum method in which they were not found by the direct smear. Eighteen specimens were found positive by the clorox method which were not

found positive by the direct smear. In six of the specimens found positive, tubercle bacilli were found only by the clorox method. None were positive by the NaOH-alum method only, but acid-fast organisms were detected in two specimens by the NaOH-alum and direct methods and not by the clorox method.

For finding some acid-fast organisms in 100 positive specimens, the clorox method proved 98 per cent efficient, the NaOH-alum method 91 per cent, and the direct smear 82 per cent efficient.

By the clorox method 68 per cent of the specimens found positive yielded the highest average count per microscopic field. By the NaOH-alum method 13 per cent and by the direct smear 6 per cent gave the highest average count per microscopic field.

In every comparison the clorox method gave more efficient results in this study than did the NaOH-alum method.

Because of the more complete digestion by the clorox method, the centrifuged sediment from positive specimens usually contained more organisms per given amount of sediment than did those obtained with the NaOH-alum method. As in previous studies when the clorox method was used, microscopic fields showed only acid-fast organisms and no visible extraneous material.

## SUMMARY

1. Every comparison in this work shows that somewhat greater efficiency for concentrating tubercle bacilli in sputum was obtained by the clorox method than by the NaOH-alum method.

2. Both the clorox and the NaOH-alum method surpass the direct smear for finding acid-fast organisms in sputum.

3. Acid-fast organisms were found in more specimens by the clorox method than by the NaOH-alum method.

4. For identical positive specimens, more than twice the average number of acid-fast organisms were found per 100 microscopic fields by the clorox method than by the NaOH-alum method.

5. The clorox method is much simpler to handle than the NaOH-alum method and gives more complete digestion, resulting in less visible extraneous material in the microscopic field.

6. For specimens containing very few organisms, a large amount of specimen may be used for concentration by the clorox method without clouding the microscopic picture.

7. When the clorox method is used, the small cotton swab should be moistened in clear blood serum before transferring the sediment from the tube to the slide. This furnishes background and fixative when there are only a few acid-fast organisms present.

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# A TRAP WITH HOLDER FOR HANDLING VICIOUS LABORATORY ANIMALS SUCH AS WILD RATS*

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THE apparatus herein described is the outgrowth of difficulty experienced in experimenting with the cotton rat (*Sigmodon hispidus*), which is not only difficult to handle, but also quite vicious, and cannot be managed like the ordinary laboratory animal.

The description of the holder employed in this apparatus is similar to the one described in a private communication† from which we quote:

> To examine their blood we have devised a cylinder of ½ inch mesh, about five inches long and from 1 to 1½ inches in diameter, closed at the one end and covered with fly screening to prevent the rat from biting through the ½ inch mesh. A piece of cardboard large enough to cover the opening of the cage should have a hole cut at the bottom to fit the open end of the cylinder. By blowing on the rat the animal will be induced to go into the cylinder. We use a cloth to hold the rat in the cylinder and find it easy to make an examination of its blood by clipping the tip of the tail.

On attempting to use this method with large individual cages, such as type L C-28‡ with sloping door, we were unable to get the animals to enter the above type of holder.

Our apparatus makes capture of the animal certain, so that it may easily be weighed, bled, or given oral or parenteral medication.

The apparatus consists of a cylindrical holder, a truncated cone-shaped tunnel or trap, and two comblike stops for immobilizing the captive. The trap is soldered to a metal shield which fits over the opening of and fastens to the animal cage. A trap door which closes the opening in the metal shield is operated by a spring catch. To the free end of the trap is soldered a metal flange over which the holder slips snugly when the animal is to be captured. The larger stop is used with the trap, the smaller with the holder.

Fig. 1 illustrates the various units of the apparatus.

## SPECIFICATIONS

The shield to which the funnel is soldered is made of 22-gauge galvanized sheet iron of sufficient dimensions to overlap the open doorway of the cage. Ours is 7½ inches high by 9 inches wide and fits well over Wahmann's standard cages L C-26 and L C-28, with openings measuring 6½ inches high by 7½ inches wide and 5½ inches by 5½ inches, respectively. The trap door is made

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‡George H. Wahmann Mfg. Co., Baltimore, Md.

of 22-gauge galvanized sheet iron and is held in place by a groove at either side of the opening in the shield. A spring catch is attached to the shield to operate the trap door.

A circular hole, 4½ inches in diameter, is cut near the bottom along the width of the metal shield so that its center is midway between the sides, leaving approximately ¼ inch of sheet iron at the bottom for soldering. The base of the trap, which is about ¼ inch larger than the opening in the shield, is soldered over the opening.

The shield, with the trap attached, placed with its flat surface over the opening in the cage makes a perfect runway from cage floor to the holder when the trap door is open.

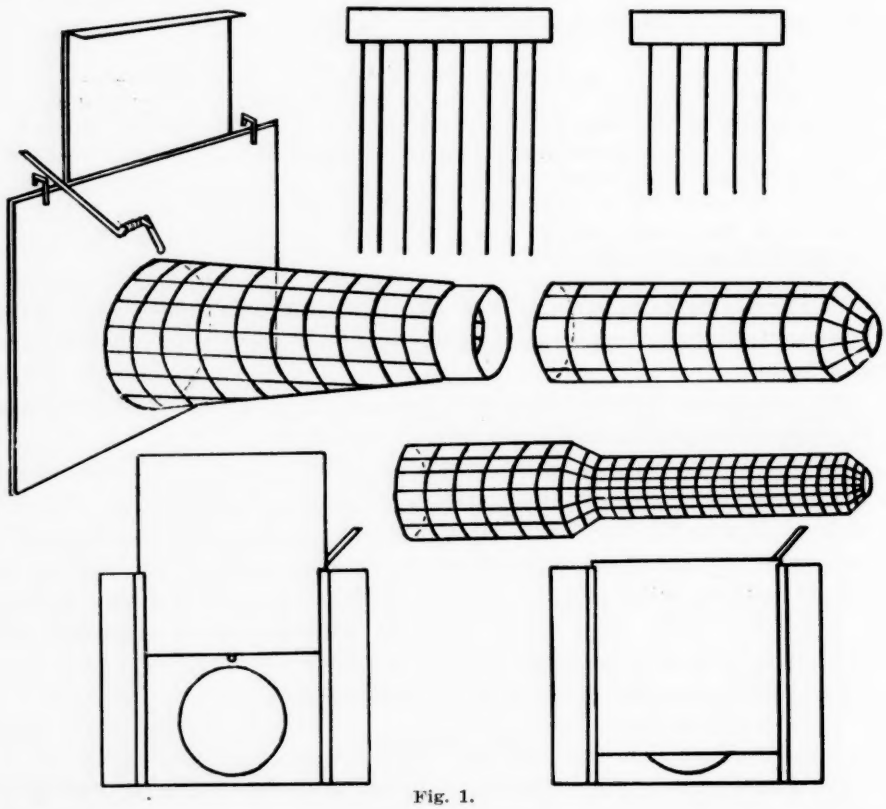

Fig. 1.

The shield is fastened to the cage by means of two pieces of No. 14 galvanized iron wire, each being approximately 2¾ inches long and fastened vertically to the upper edge of the front of the shield (the side to which the trap is fastened) about 1 inch from each end with approximately 1½ inches being soldered to the base. They are then bent to a right angle over the edge of the base and again at a right angle about ¾ inch from each end thus making hooks that fasten over the upper framework of the cage, securely fastening it to the open doorway.

The trap is a truncated cone of 18-gauge, 4-mesh hardware cloth. It is 7½ inches long with the inside diameter at the base being 4¾ inches and that at the top, 2½ inches.

Soldered to the inner part of the small end to a depth of about ½ inch is a galvanized sheet iron flange 1¾ inches long and made to taper slightly so that the free end measures about 2 inches outside diameter and over which the large end of the holder fits tightly.

The holder is made of hardware cloth similar to that used for the trap. It is a cylinder 9 inches long and 2¼ inches inside diameter. One end is slightly tapered 1¼ inches from the opening to form a circular opening ¾ inches in diameter and is made secure by a ring of wire soldered around the entire circumference. We also have other holders with inside diameters of 1¾ inches, 1½ inches, and 1¼ inches. See Fig. 1 showing the method of reducing the diameter so that various-sized animals may be accommodated.

The stops are of two sizes but are of similar material. As mentioned, they are shaped like a comb, the base being made of 1/16 inch by ¾ inch flatiron and the teeth of 1/16 inch welding rods, which are soldered upon the flatiron.

The larger comb has a base four inches long, with eight rods 4¾ inches long soldered perpendicular to the base in parallel. Distance between rods is not equal but as follows: ¼ inch, 7/16 inch, ½ inch, 7/16 inch, 7/16 inch, and ¼ inch. The first and last rods are soldered about ½ inch from each end of the base. The unequal distance makes it possible to thrust the rods through the mesh in the tapering cone when trapping the animal.

The small comb has a base 2½ inches long with five rods 3½ inches long soldered similar to the large one but beginning about 7/16 inch from one end and spacing all ½ inch apart, variability in this respect not being necessary since the holder in which it is used is a cylinder.

## CAPTURING THE ANIMAL

To capture the animal, the apparatus is assembled as described. The shield with the open trap and holder attached is fastened to the open doorway of the cage. By blowing upon the animal from the rear of the cage, it will usually seek the opening and enter the trap. As soon as the animal enters the trap, the lever attached to the spring is moved by slight pressure of the forefinger which releases the trap door, thereby preventing re-entrance into the cage. The large stop may be used to induce the animal to move to the holder, and, as it enters, the smaller stop is similarly used, forcing the animal to the extreme tip where it is firmly held by the stop which has been successively moved behind it as it moves forward. The diameter of the cylinder is so small that the animal cannot turn around.

The holder containing the animal is then removed from the trap by slipping it off the smooth metal flange. The animal is not injured by the apparatus as it may be if tongs or similar instruments are used for catching and holding. The holder with the stop in place is simply turned over so that the flat side of

the stop is down while the animal is weighed. The flat surface prevents the apparatus from rolling off the balance.

Either the opening at the end of the holder or a mesh of the hardware cloth may be used to pass a tuberculin syringe through to feed the animal a definite amount of a substance. It is possible to inject an animal by various routes when it is in the holder or to take blood samples from the heart, tail, or ears, or by clipping off a toenail.

We feel that the apparatus described herein is especially convenient and useful for handling rodents which have a tendency to bite when picked up by hand.

# A LABORATORY HOLDER FOR IMMOBILIZING EXPERIMENTAL RATS

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IN THE pharmacologic study of ointments and other drugs in this laboratory, it was deemed desirable to study their effects on the albino rat. The difficulties of immobilizing these animals for extended periods of time to prevent contamination by licking and the eating of feces, and to prevent injury to the animals or to the site of application, were overcome by the design of a holder to be described. The usual animal board was found unsuitable for our purposes. Elefthériou[1] described an apparatus in which the animals were laced into a cloth apron supported between two metal frames, and Lecloux[2] described a similar apparatus for mice. Neither of these, however, allowed for the feeding and watering of the animals during the period of confinement.

The holder herein described is easily constructed of readily available materials. Details of the holder are shown in Fig. 1. The neck and hip stocks were made of sole leather approximately $\frac{3}{16}$ of an inch thick, and the locks were tin plate cut from a tin can. The locks were secured at the hinge end with copper harness rivets and were fastened with small brass bolts $\frac{1}{8}$ of an inch in diameter and of suitable length. The dimensions shown are not critical but were found suitable for a rat weighing 250 grams. The stock openings can be made larger or smaller to accommodate the size of rat desired. The size of the holder shown has been used for smaller rats by padding the upper part of the openings with cotton wads held in place with adhesive tape. Care should be taken, however, that the space between the bottom of the opening of the hip stock and the floor of the cage be not too great or injury to the animal may result. The bars separating the stocks were made of brass rods $\frac{3}{16}$ of an inch in diameter threaded at each end. One end of each is threaded for two inches to allow for adjustment to the length of the body of the rat.

In use, the entire holder is fastened to the bottom of a regular wire-mesh individual steel cage by means of small 12-gauge steel wire hooks. In Fig. 2 is shown such an arrangement with an animal in place, the side of the cage being bent back for a better view. A battery of holders could be fastened to a board to enable simultaneous observation of the animals. Food and water can be supplied to the animals in the cages in the usual manner.

Although the rat can be put into the holder by two operators without anesthesia, the latter generally has been used. The animal is lightly anesthetized with ether and after opening the locks, the flexible leather stocks are bent aside to permit the entrance of the neck and hips. The locks are then closed by means of the bolts and the holder fastened to the bottom of the cage.

For most of the experiments, the animals were thus confined from one to twenty-four hours with a maximum of seventy-two hours. The animal rests

From the Division of Pharmacology, Food and Drug Administration, Federal Security Agency.
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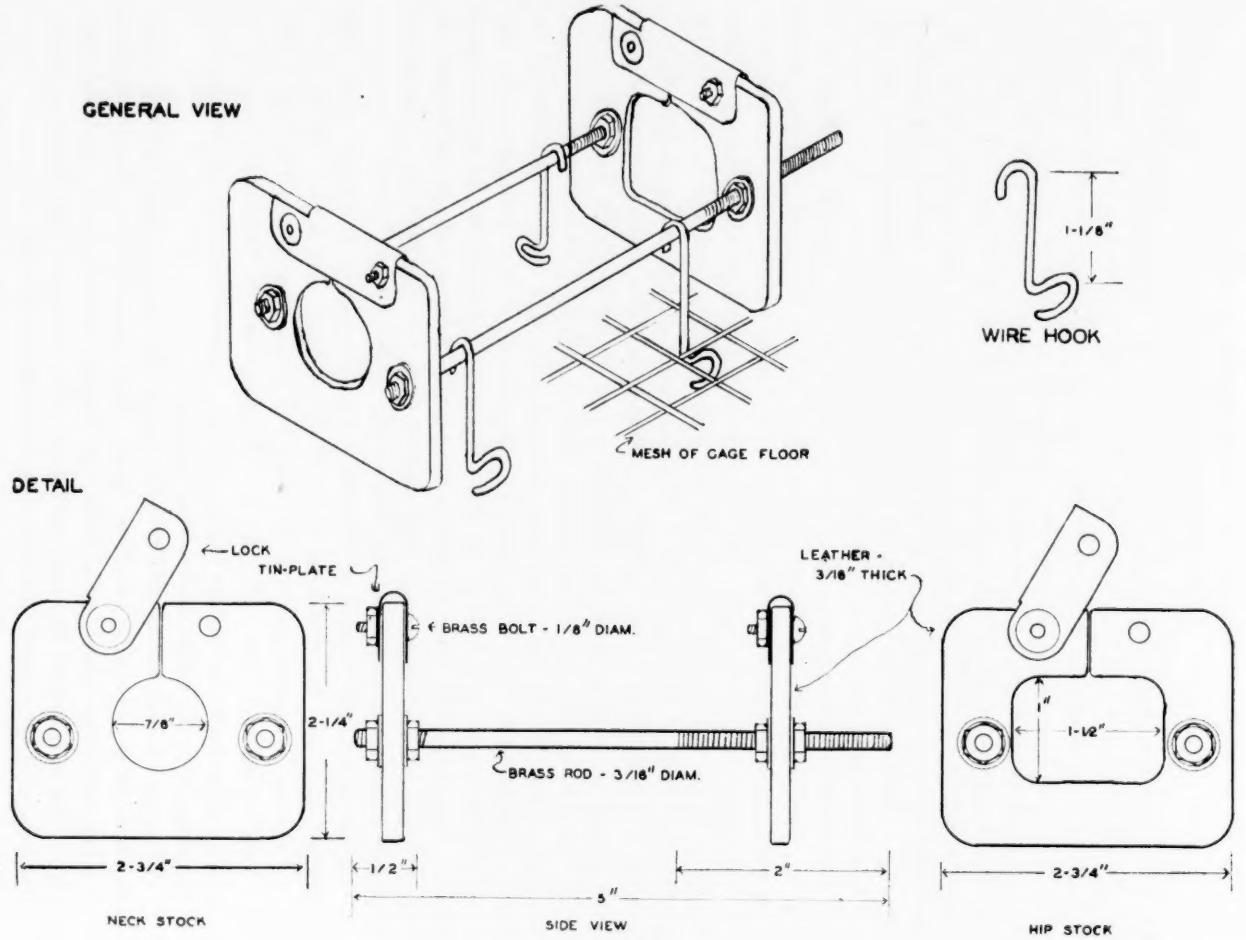
GENERAL VIEW
MESH OF GAGE FLOOR
1-1/8"
WIRE HOOK
DETAIL
LOCK
TIN-PLATE
BRASS BOLT - 1/8" DIAM.
LEATHER - 3/16" THICK
7/8"
2-1/4"
BRASS ROD - 3/16" DIAM.
1"
1-1/2"
2-3/4"
1/2"
2"
5"
2-3/4"
NECK STOCK
SIDE VIEW
HIP STOCK

Fig. 1.

comfortably on the bottom of the cage, but occasionally the eyes of animal may appear congested due to struggling.

In order to gain some idea of the effect of confining the animals for periods as long as seventy-two hours, the following experiment was performed. Eight female rats averaging 250 grams were selected. All were lightly anesthetized with ether, placed in separate cages, and given a measured, ample amount of food and water. Four of the animals were confined in the holders. The animals were weighed daily. After seventy-two hours, the control rats had gained 2 per cent and the confined rats had lost 10.5 per cent of their initial weight.

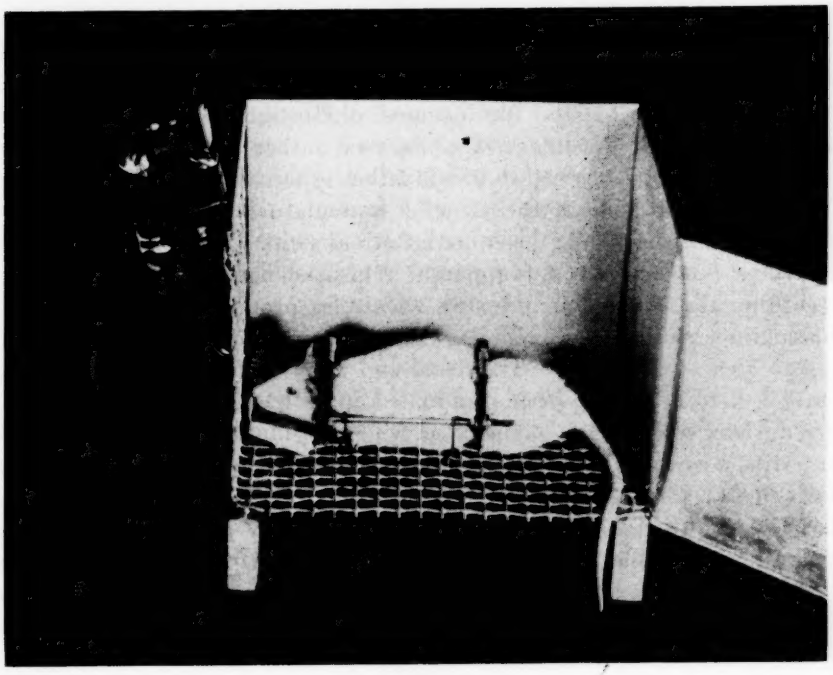

Fig. 2.

The confined animals were now removed from the holders and given ample food and water, while the animals which had been used for controls were deprived of all food and water. After an additional seventy-two hours (one hundred and forty-four hours from the beginning of the experiment), the control animals had lost 18.8 per cent of their weight, and the previously confined animals had gained 15.7 per cent, or a gain of 3.6 per cent over the one hundred and forty-four hour period. The animals appeared normal in every respect. It is apparent that while the animals are somewhat ill at ease while confined and lose some weight during that time, recovery is rapid and complete. The records show that the confined animals ate and drank about one-half as much as the control animals.

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1. Elefthériou, D. S.: Un nouvel appareil de contention pour rats, J. de physiol. et path. gén. **29**: 514, 1931.
2. Lecloux, L.: Appareil simple pour contention prolongée de petits animaux, Bull. d'histol. appliq. à la physiol. **4**: 403, 1927.

# A NOTE ON THE UNSUITABILITY OF GUINEA PIG JEJUNUM FOR STUDY BY THE MAGNUS METHOD

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BERNHEIM* has made the interesting and rather curious observation that pilocarpine will relax the contraction induced by histamine in isolated strips of the jejunum of the guinea pig.

We have found that records of the contractions of the jejunum of guinea pigs, made by the method of Magnus, indeed appear to show that marked relaxation of histamine spasm is produced not only by pilocarpine, but also by other parasympathomimetic agents; for instance, physostigmine and acetyl-β-methylcholine. However, if the intestine is observed rather than the recording lever, it is found that what is recorded as relaxation is actually a response to a powerful wave of contraction of the circular musculature of the jejunum, a phenomenon which actually lengthens the intestinal strip.

Further evidence that the apparent relaxation is artefactual was obtained by recording the changes in pressure which are produced within the jejunum by histamine and by pilocarpine. The proximal end of a strip of jejunum from 4 to 5 cm. was tied off. The distal end was tied to a small cannula, which was attached to a 1 c.c. pipette, graduated in 0.01 c.c. The jejunum was suspended in 50 c.c. of warm, oxygenated Ringer's solution. The pressure inside the intestine was recorded by noting the variations in level of the Ringer's solution within the pipette. A typical experiment showing maximal and minimal levels of the solution, as affected by histamine and by pilocarpine. is presented in Table I. (Since the pipette is read from 0 above to 1.00 below, a decrease in the readings accompanies a rise in the fluid column and therefore corresponds to an increase of pressure within the intestinal segment.)

TABLE I

| | MAXIMAL LEVEL (C.C.) | MINIMAL LEVEL (C.C.) |
|---|---|---|
| Before addition of drug | 0.77 | 0.79 |
| After addition of histamine (1:50,000) | 0.50 | 0.53 |
| After addition of pilocarpine (1:50,000) | 0.38 | 0.40 |

Thus, following the contraction induced by histamine, pilocarpine induces a further increase in the pressure inside the jejunum, even though the length of the intestinal segment may be increased.

It is concluded that the jejunum of the guinea pig, at least for some purposes, is quite unsuitable for studies by the method of Magnus. This deficiency is due to the presence of powerful circular muscle fibers, the contractions of which may overshadow contractions of the longitudinal fibers.

From the Wyeth Institute of Applied Biochemistry.
Received for publication, Nov. 19, 1945.

*Bernheim, F.: Interaction of Pilocarpine and Histamine on the Intestine, J. Pharmacol. & Exper. Therap. 43: 509, 1931.

# PHOTOMICROGRAPHY: A SHORT METHOD USING NEGATIVE PROJECTION PRINTS

DONALD W. LEONARD, M.D., F.A.C.S.
CAMBRIDGE, N. Y.

THE pages of medical periodicals and textbooks testify to the educational value of photomicrographs in the demonstration of tissue structure and changes resulting from disease. Rarely, if ever, does the average physician think of preparing such prints for himself; however, in spite of the fact that the value of the photomicrograph is so generally understood, relatively few of the smaller clinical laboratories are prepared to make such prints for him. There are many reasons for this, the most obvious being the fact that a special type of fairly expensive apparatus and a complicated technique must be available. Such equipment, because of its limited usefulness in a small clinical laboratory, is usually considered a luxury and no photomicrography is therefore available. This difficulty will continue to exist as long as physicians adhere to the unreasonable belief that the finished photomicrograph must show the field *just as the pathologist sees it through his microscope.* It has been assumed that to be acceptable the end product must be a positive print made from a negative which has been exposed in a camera-like arrangement which holds the film in focus over the microscope. However, if it is remembered that the fundamental purpose of medical illustration is the clarification of points emphasized in the written text, it may seem to be quite legitimate to question the absolute need for adherence to this traditional form of photomicrography. It is conceivable that some other type of reproduction will make microscopic details just as clear to the reader.

Several months ago I was forced by the absence of any apparatus for standard photomicrography to make a decision on this point and to improvise a technique which, as results were reviewed, seemed to have several merits beyond an obvious simplicity. A case of fatal dissecting aneurysm of the thoracic aorta, of well-authenticated traumatic origin, came to autopsy at the Mary McClellan Hospital and, as a rare example of this disease, the case history was prepared for publication with a discussion of the literature. Severe histologic sections of the injured aorta were considered instructive enough to be used. Two photomicrographs were made from the stained slides in the form of negative paper prints by projection, and these hybrid photomicrographs proved acceptable as illustrations in a previously published article.*

In spite of the fact that it has been impossible to find any medical article using or describing negative projection prints of this type, no claim will be made that medical illustrators have never before considered the idea. If negative prints have been suggested, it is more than probable that medical authors

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*Leonard, D. W.: Dissecting Aneurysm of the Thoracic Aorta Due to Trauma, Am. J. Surg. 69: 344-351, 1945.

themselves have been the ones to discard what appeared to them to be distortions of real microscopic anatomy. If this has been the case, it is unfortunate that someone did not point out that whatever "distortion" resulted from the transposition of density values from positive to negative might have been improvements in the visualization of the tissue and that the change could not have been any more artificial than the already accepted artifacts of staining. The change might also have actually been more "true" in relation to living tissue than any other kind of photography.

## MAKING PROJECTION PRINTS

Simplicity is the first advantage to be credited to a technique which makes prints on paper directly from the microscope slide. It is a darkroom technique but, in this respect, it is essentially the same as the step that has been used in the standard routine to get paper prints from negatives. It uses the slide in place of the negative and the microscope as the enlarger. Most teachers of pathology have at one time or another used a microscope as a means of projecting the enlarged image of a tissue section onto a screen for class demonstration. To make a projection print, it is necessary only to arrange the micro-

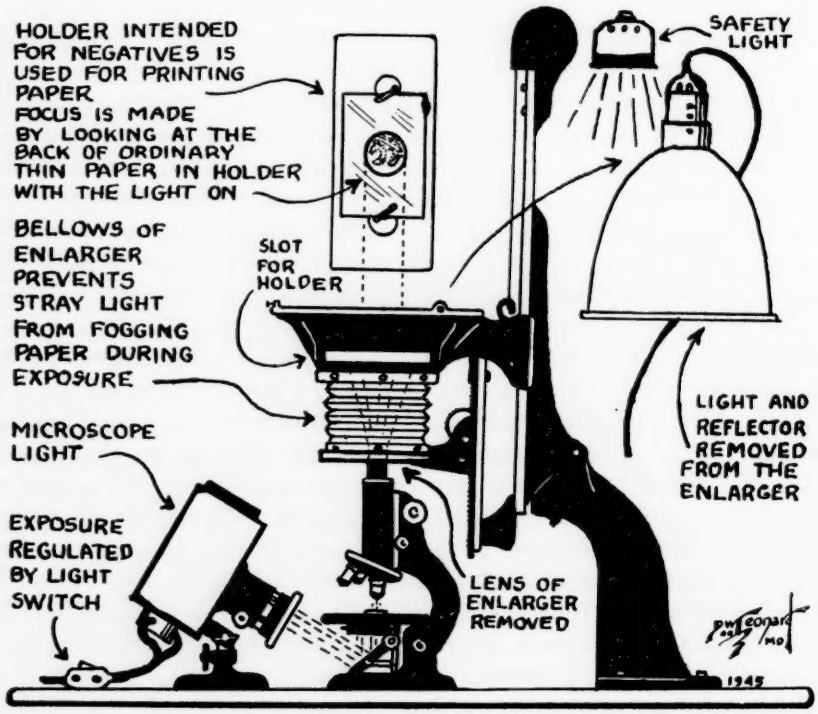
HOLDER INTENDED FOR NEGATIVES IS USED FOR PRINTING PAPER
FOCUS IS MADE BY LOOKING AT THE BACK OF ORDINARY THIN PAPER IN HOLDER WITH THE LIGHT ON
BELLOWS OF ENLARGER PREVENTS STRAY LIGHT FROM FOGGING PAPER DURING EXPOSURE
MICROSCOPE LIGHT
EXPOSURE REGULATED BY LIGHT SWITCH
SLOT FOR HOLDER
SAFETY LIGHT
LIGHT AND REFLECTOR REMOVED FROM THE ENLARGER
LENS OF ENLARGER REMOVED
1945

Fig. 1.—Assembly of apparatus used for taking the photomicrographs reproduced with this discussion. Although the image of a tissue section may be projected directly onto printing paper tacked to a board, it is safer to make use of some arrangement like the bellows of an enlarger as a light-trap to protect the paper from the stray light that will unavoidably leak from any microscope lamp. A commercial photographer may combine his enlarger with the doctor's microscope, if the latter cannot himself use this piece of photographic equipment.

scope, the slide, and the light source so that the image will be projected onto the photographic paper. For medical illustrations, a moderate enlargement is all that usually will be desired so that the suitable distance from the ocular of the microscope to the paper will seldom be more than a few inches. The entire assembly may be quite compact. The advantage of a scheme which obtains a finished picture in a single step will occur to anyone and the technical details of the process will be obvious to any physician having some knowledge of photography. It will not be necessary in this discussion to do any more than illustrate a simple arrangement of my equipment (Fig. 1) and to demonstrate by examples that the essential features of a tissue section can be shown as well in a negative print as in any other photomicrograph (Figs. 2 to 5).

When an individual has assembled a combination of his own available equipment, he will have to standardize certain values, the first being the strength of his light source as it effects the length of exposure which will be required for each magnification and for slides which vary in their staining. A few trial prints should establish these values. Projection printing paper registering various degrees of contrast may be tried, but sharp contrast paper (No. 3) with a glossy finish will usually be the choice. The framing of the pictures takes care of itself because the field selected will be framed automatically in the circle of the microscope lens. After the exposure has been made, the steps in development will be the same as those of normal photography.

## INTERPRETATION

My experience with negative prints has already shown that the usual reaction of the medical observer is instant interest in the clear details and an attempt to identify the tissue while completely unaware of the reversal of tone values this type of photomicrograph presents. A pathologist's more discerning inspection, of course, discovers the reversal with involuntary resentment or curious pleasure, according to that individual's attitude toward innovations. For his benefit it may be argued that space is empty and dark and that a section of tissue may be mounted like a lace curtain in front of this empty background. The resemblance to objects viewed under dark field becomes striking and one may become enthusiastic with the realization that he has discovered a picture which appears just as he had imagined the original, unstained tissue section must have looked as it came off the microtome knife.

From such photomicrographs it should not be difficult to demonstrate the changes caused by disease and to correlate these changes with the descriptions recorded by those who have studied the same specimens directly under the microscope.

## SUMMARY

Out of the need for self-made photomicrographs has developed a suggestion that any medical author can resort to the use of a negative projection print whenever he needs a photomicrograph for illustration. To the obvious advantage of requiring no more than the equipment already available can be

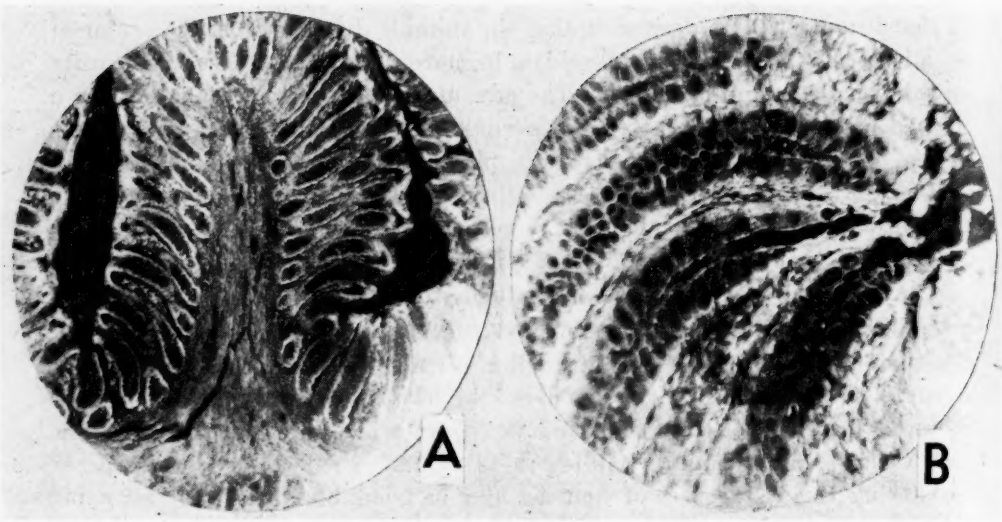


Fig. 2.—*A*, Large intestine. Mucous, submucous, and muscular coats are all distinguishable. The crypts are for the most part cut longitudinally and the secreting elements are outlined in white by the cell nuclei near the basement membrane. *B*, Large intestine. High power magnification of the crypts to demonstrate the detail of the mucus containing goblet cells.

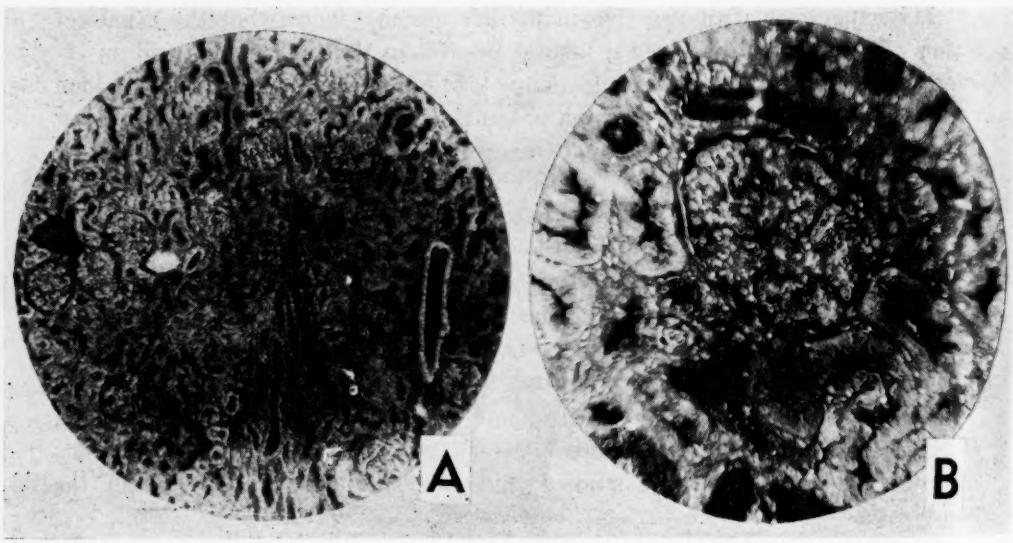


Fig. 3.—*A*, Kidney. A demonstration of the medullary structure of a normal kidney with several glomeruli in their capsules surrounded by cut sections of convoluted tubules. The field also includes some collecting tubules and a diagonal section through at least one fairly large blood vessel. *B*, Kidney. A higher magnification of a glomerulus in its capsule. Characteristic cell lining is evident in the neighboring convoluted tubules.

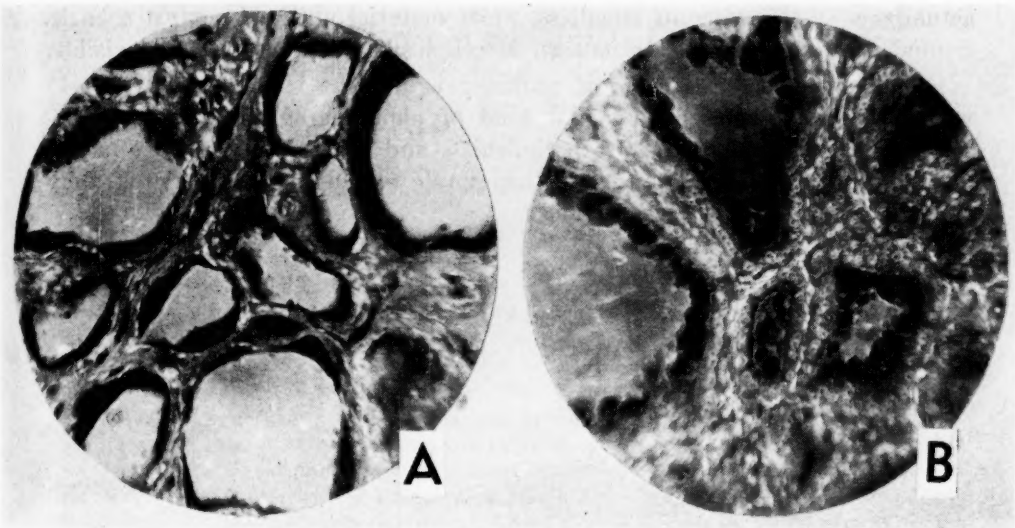


Fig. 4.—*A*, Normal thyroid. Section shows the typical arrangement of follicles, each well filled with homogeneous colloid substance and lined with a layer of flat epithelial cells. *B*, Toxic goiter. Hyperplasia is evidenced by the change of the epithelial cells to the characteristic columnar type. The entire tissue is much more cellular, but this example is not extreme because there is still a good deal of colloid substance and very little irregularity of the follicle outlines.

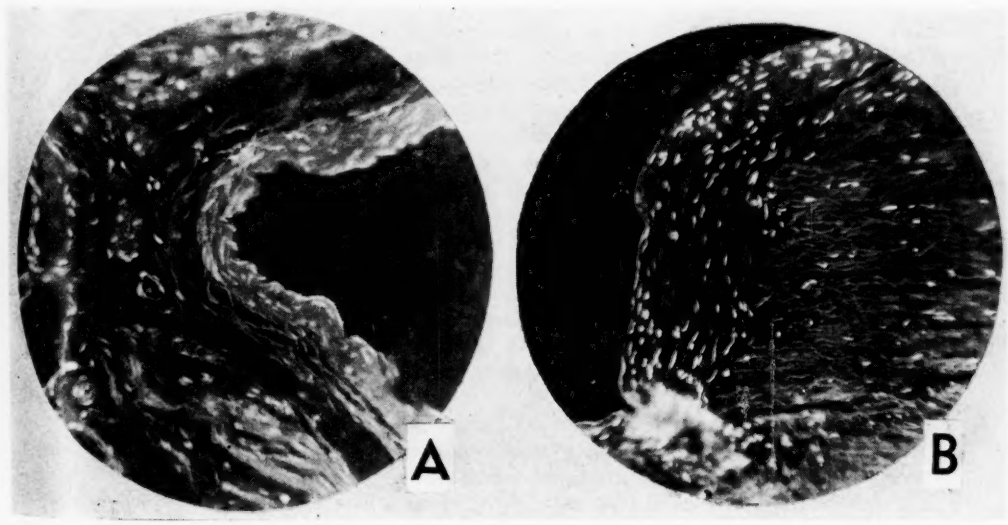


Fig. 5.—*A*, Subcutaneous vein. Section shows a portion of a fairly large vein embedded in loose connective tissue. The intima can be identified, but the media and the adventitia are indistinguishable. *B*, Thoracic aorta. This section is taken from the descending portion of the aortic arch at the point where the initial rupture of the intima and the media started a dissecting aneurysm. The elastic fibers of the media have retracted, allowing the cells of the intima to cover their ends.

added the claim that such a print will show a tissue section more nearly as it actually is—a thin slice of bloodless, white material outlined against a background of space, its delicate pattern of cell walls and nuclei sharply visible.

One improvised assembly of ordinary equipment is presented (Fig. 1) with several examples of this new kind of photomicrography made as described. It is hoped that more pathologists and editors will approve of the positive projection print and give this simple method of photomicrography official sanction for more extensive use.

# BOOK REVIEWS

**Pathology of Tropical Diseases.** By *J. E. Ash,* Colonel, M.C., U.S.A.; Director, Army Institute of Pathology, Army Medical Museum; and *Sophie Spitz,* M.D., C.S., A.U.S.; Pathologist, Army Institute of Pathology, Army Medical Museum. W. B. Saunders Company, Philadelphia. Price $8.00. Cloth with 350 pages and 941 illustrations.

In no field of medicine is it more necessary for texts, monographs, and journal articles to carry good illustrations than in pathology. Hence there are two kinds of books in pathology, those with good illustrations and those with poor illustrations. The illustrations in this atlas are not good—they are superb. The authors have exercised good judgment in selection of subjects; Mr. Reeve and the staff of the Photographic Laboratory of the Army Institute of Pathology have outdone themselves in technical details; and the publishers have faithfully reproduced the originals.

Added value is given to the illustrations by the explanatory legends. The usual custom in texts is to append only a simple statement of the diagnosis. In this manual the legends are skillfully combined to form a brief exposition of the disease. For example, on pages 166, 167, and 168 there is a section on North American blastomycosis. The legends of the nine pictures read successively: "(1) Primary cutaneous blastomycosis causes papulopustular lesions with numerous small satellites surrounding the large discharging ulcers. The pitted scars are residium of smallpox. (2) Healing leads to formation of crusts and atrophic scars in the center of the lesions while irregular extension of the process continues at the periphery thus accounting for the characteristic clusters. (3) Systemic blastomycosis involves the lungs more frequently than any organ, other than skin. The involvement is of diffuse miliary type, grossly indistinguishable from miliary tuberculosis. (4) Spleen is commonly involved and shows a few or many pale caseous foci. (5) Bone lesions may be osteolytic abscess cavities lined by pyogenic membrane. (6) A characteristic of primary cutaneous blastomycosis is the extreme pseudo-epitheliomatous hyperplasia with many ramifying epidermal extensions into dermis. The cavities thus formed are filled with purulent exudate and epithelial debris. The granulomata in the dermis are usually discrete. Numerous intra-epidermal and intradermal abscesses are present in this field. (7) Organisms *(Blastomyces dermatitidis)* are frequently found within giant cells of the granulomatous lesions of the dermis or within the intradermal abscesses. The fungi occur in tissue as single or budding thick-walled, double-contoured cells. (8) Distribution of pulmonary lesions is of a generalized, hematogenous type. Larger pneumonic areas may result from confluence of the miliary nodules. There is superficial resemblance to miliary tuberculosis. Note the necrotic center of many of these tubercles. (9) Granulomatous nodules show numerous multinucleated giant cells within a discrete collection of epithelioid cells and lymphocytes. Giant cells of the granulomata may contain the double contoured cell of *Blastomyces.* Endospores are always absent in contrast with *Coccidioides immitis.*" This is a short but entirely adequate description of the salient features of the disease.

Each chapter, and there are twenty-two, has an introductory discussion of the diseases included, with a paragraph on definition, clinical features, and pathology. In the case of some, there is also a paragraph on epidemiology. In all, seventy-five disease entities are considered ranging from yellow fever to pinta, to heat exhaustion, to beriberi, to ainhum. There are a few selected references at the end of each chapter, and they are to recently published articles almost exclusively. For example, in Chapter 1, the dates are 1945, 1943, in press, 1944, 1944, 1944. Thus, only one is two years old and one is in the future.

Maps and diagrams are used to advantage to show geographic distribution and to clarify the stages in the life cycles of many of the metazoan parasites.

It is to be hoped that this book may be the first in a new viewpoint of American medical publishers. Frequently, one picture is worth more than hundreds of words. **Medi-**

cine should be an objective, documented science. In the nineteenth century many texts and atlases of pathology with excellent black and white and colored illustrations were published. Some authors cut their own lithograph plates. Perhaps a way can be found in the economically complex twentieth century to revive the practice of printing richly illustrated books. This atlas of Ash and Spitz proves that the authors, photographers, artists, and engravers are fully ready and that the publishers are willing.

ROBERT A. MOORE.

**Diseases of the Breast.** By *Charles F. Geschickter,* M.A., M.D., Lt. Comdr., M.C., U.S.N.R.; Director of the Francis P. Garvan Cancer Research Laboratory; Pathologist, St. Agnes Hospital, Baltimore, Md.; with a special section on treatment in collaboration with *Murray M. Copeland.* Second edition, J. B. Lippincott Company, Philadelphia, Pa., 1945. Price $12.00. Cloth with 826 pages and 593 illustrations.

The second edition of this book presents a definite improvement over the first in many details. The fundamental treatment of the subject, however, has not been essentially altered.

The chief appeal of this monograph rests in its biological approach to the subject. This has been competently handled. The normal development of the breast is considered in relation to the ovarian and hypophysial hormones and their effect upon normal growth and development. The estrogen-progesterone balance in the production of normal mammary structure is well considered. The anatomic and clinical disabilities that result from an escape from this normal balance has been used as the basis of the understanding of the cystic and hyperplastic changes so often encountered in the human breast. An effort is made to divide these lesions into several subgroups based upon their pathologic and clinical features as well as urinary assays and animal experimentation.

In these subgroups, certain minor criticisms might well be raised. For instance, one would question the consideration of mastodynia, or painful breast, as a pathologic entity. One would also wonder why the lesions of fibroadenoma and intracystic or intraductal papilloma are considered as neoplasms when they so often demonstrate their endocrine origin. The estrogen-progesterone studies that have been made on clinical lesions have been excellently portrayed. It is unfortunate that more of them are not available.

The statistical studies on the relationship between chronic cystic mastitis and cancer of the breast are well presented. While not an exhaustive review of the literature is offered, enough important papers are considered to make this a fair consideration of the subject. Bleeding from the nipple is considered separately. In view of the fact that in the author's series such a high percentage (seven) of patients with bleeding from the nipple subsequently developed cancer in that breast, one finds reason to question the conservative treatment that he has to offer for this lesion. One would also find reason to question the effect of hormonal therapy suggested in various places in the monograph on the basis of the evidence presented.

In the section related to malignant mammary tumors, those factors influencing the development and prognosis are well considered. The rather loose division into infiltrating adenocarcinoma and circumscribed adenocarcinoma probably is well worth while from a prognostic standpoint. The clarity of this classification is obscured somewhat by the inclusion of so many subgroups related chiefly to their microscopic appearance. Such detailed morphologic classification of cancer is usually more confusing than enlightening.

Once again an effort to prognosticate what will happen to a particular patient with cancer of the breast, based solely on the microscopic appearance of the lesion, remains unconvincing. This reviewer notes with pleasure that Paget's disease of the nipple is considered as primary cancer of the ducts.

The surgical and radiologic treatment is adequately handled. The format of the book is excellent, the type pleasing to the eye, most of the illustrations well reproduced, and typographic errors rare. Both the author and the publisher deserve praise for this monograph.

NATHAN A. WOMACK.